CYANOBACTERIA-GRAZER INTERACTIONS:

CONSEQUENCES OF TOXICITY,

MORPHOLOGY, AND GENETIC DIVERSITY

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SUMMARY

Interactions between cyanobacteria and herbivorous grazers play an important role in mediating the responses of freshwater phytoplankton assemblages to nutrient enrichment and top-down manipulation. Negative consequences associated with these interactions include dangerous blooms of harmful blue-green algae that have been implicated in the sickness and death of fishes, livestock, and, in extreme cases, humans. Frequently cited mechanisms influencing the interactions between grazers and cyanobacteria include cyanobacterial toxicity and morphology. I used meta-analysis and laboratory experiments to determine the effects of cyanobacterial toxicity and morphology on the fitness of freshwater zooplankton.

In Chapter 1, I used two meta-analyses to quantitatively synthesize the available literature on zooplankton-cyanobacteria interactions in order to tease apart the mechanisms responsible for the poor food value of cyanobacteria for zooplankton – namely the effects of cyanobacterial toxicity and morphology. The primary analysis compared zooplankton population growth rates for grazers fed diets containing good foods, such as chlorophytes and flagellates, versus grazers fed diets containing all or part cyanobacteria. Results from this analysis show that (1) cyanobacteria are poor food for grazers, (2) grazers are inhibited similarly by toxic and non-toxic cyanobacteria, (3) cladocerans and rotifers are affected by cyanobacteria, in general, and (4) diets containing filamentous cyanobacteria are less inhibitory to grazers than diets containing single-celled and colonial cyanobacteria. Since I did not find an effect of cyanobacterial toxicity in the primary analysis, I conducted a secondary analysis where I compared population growth (calculated using survivorship curves for most studies) for starved

grazers versus grazers fed diets containing all or part cyanobacteria. Results from this analysis show that grazers are affected differently by toxic and non-toxic cyanobacteria. Non-toxic cyanobacteria are a significantly better food source for grazers than being starved, while toxic cyanobacteria are a significantly worse food source for grazers than being starved. The latter effect is consistent with the definition of "toxic" for cyanobacteria initially proposed by Winfred Lampert in the early 1980's. The discrepancy between the results for the primary and secondary analyses for the effects of cyanobacterial toxins appears to be driven by one single-celled, toxic culture collection strain of *Microcystis* used in many of the experiments incorporated into the secondary analysis. This strain of *Microcystis* produced extraordinary negative effects on grazers. Thus, the toxicity effect observed in the secondary analysis may not be a general phenomenon, but instead an experimental artifact suggesting that the literature may not be fully appreciating the diversity of cyanobacteria in nature.

In Chapter 2, I surveyed 14 lakes throughout the lower peninsula of Michigan to determine the amount of genetic variation within and among populations of *Microcystis aeruginosa*. Between 40 and 80 *Microcystis* colonies from each lake were isolated into a nutrient rich medium and maintained in an incubator chamber. Isolate survivorship was poor (<10%), but was positively correlated with lake phosphorus levels. Fifty-three of the 67 genetically analyzed isolates were shown to be genetically distinct. Consequently, we found significant genetic variation across and within lakes. All analyzed populations were shown to contain strains of *Microcystis* that are toxigenic (i.e., capable of producing microcystins), while 4 of the populations also contained strains of *Microcystis* that lack the genetic machinery to produce microcystins.

In Chapter 3, I used a 9 day laboratory growth rate experiment to determine the magnitude of variation in maximum population growth rate and morphology across 32 distinct *Microcystis aeruginosa* genotypes. Growth rate, cell size, and colony surface area were shown to be different across the strains, while growth rate and colony surface area were also shown to differ across populations. A counter-intuitive positive correlation was found between colony surface area and growth rate, while no relationship was found between growth rate and microcystin concentration for toxigenic strains.

In Chapter 4, I used several 12 day laboratory feeding experiments incorporating freeze-dried *Chlorella* coated with solvents to test for the effect of the crude lipophilic extract from a toxic strain of *Microcystis aeruginosa* and the effect of the cyanotoxin, microcystin-LR, on the fitness of two *Daphnia pulicaria* genotypes that vary in their response to a diet containing toxic *Microcystis*. Interestingly, the *Daphnia* most inhibited by *Microcystis* was not harmed by the addition of microcystin-LR, while the *Daphnia* least affected by *Microcystis* showed large negative fitness effects when fed diets containing freeze-dried foods treated with solvent and microcystin-LR. Neither *Daphnia* was harmed by the lipophilic extract of *Microcystis*, and one *Daphnia* even shown enhanced population growth on the diet supplemented with the lipophilic chemistry of *Microcystis*.

Together, results from these studies indicate that cyanobacterial toxicity and morphology can influence the fitness of herbivorous grazers; however conclusions drawn from these studies were heavily influenced by which herbivore genotype was considered. Thus, genetic variation within aquatic communities can have significant ecological implications on the promotion and/or control of harmful cyanobacterial blooms in nature.

CHAPTER 1

EFFECTS OF CYANOBACTERIAL TOXICITY AND MORPHOLOGY ON THE POPULATION GROWTH OF FRESHWATER ZOOPLANKTON: META-ANALYSES OF LABORATORY EXPERIMENTS

Abstract

We synthesized data from 66 published laboratory studies, representing 597 experimental comparisons, examining the effects of cyanobacterial toxicity and morphology on the population growth rate and survivorship of 17 genera (34 species) of freshwater, herbivorous zooplankton. Two meta-analyses were conducted with these data. The primary analysis compared herbivore population growth rates for grazers fed treatment diets containing cyanobacteria versus control diets comprising phytoplankton that are generally considered to be nutritious for zooplankton (chlorophytes and/or flagellates). This analysis confirmed that cyanobacteria were poor foods relative to small chlorophytes and flagellates. More importantly, filamentous cyanobacteria were found to be significantly better foods for grazers than single-celled cyanobacteria over all studies. Surprisingly, the presence or absence of commonly-measured toxic compounds (microcystins in 70% of the cases) in the diet had no overall influence on grazer population growth relative to control diets. A secondary analysis compared survival rates for grazers fed cyanobacteria versus no food. In contrast to the primary analysis, grazer survival was more negatively affected by toxic cyanobacteria than non-toxic cyanobacteria, relative to starvation. However, this difference was attributable to the

effects of a single *Microcystis* strain, *Microcystis,* PCC7820. Thus, though some cyanobacterial strains appear to be toxic to some strains of zooplankton, the overall role of commonly-assayed cyanobacterial toxins as a determinant of food quality may be less than widely assumed. We suggest that more attention be focused on nutritional deficiencies, morphology, and the toxicity of undescribed cyanobacterial compounds as mediators of the poor food quality of cyanobacteria for zooplankton.

Introduction

Interactions between bloom-forming cyanobacteria and zooplankton are central to the responses of freshwater phytoplankton assemblages to nutrient enrichment and top-down manipulation (Burns 1987; Lampert 1987; Sommer 1989). Consequently, a large body of research has been directed at understanding mechanisms by which cyanobacteria affect zooplankton and vice versa. This information has important implications for understanding planktonic community structure and function, as well as water quality, in lakes. For example, the filamentous morphology of certain cyanobacteria has been shown to negatively affect large cladocerans more than small cladocerans through reduced fecundity (Webster and Peters 1978; Gilbert 1990), a mechanism that may help to explain declines in the dominance of large cladocerans with lake eutrophication (Jeppesen et al. 2000). In addition, cyanobacteria produce secondary metabolites that can cause illness, and in some cases death, of livestock and humans exposed to water with high cyanobacterial concentrations (Carmichael et al. 2001). If these metabolites are similarly toxic to co-occurring zooplankton, the potential for stimulation of toxin production via interactions between cyanobacteria and zooplankton could have implications for public health. Given these considerations, there is considerable value in deciphering the specific mechanisms of interaction between cyanobacteria and zooplankton.

It is well established that bloom-forming cyanobacteria are, in a general sense, of poor food quality for herbivorous zooplankton (Porter and Orcutt 1980). Three properties of cyanobacteria have been proposed to account for this poor quality - filamentous/colonial morphologies, production of intracellular secondary metabolites

with toxic properties, and deficiencies in essential nutrients (Porter and Orcutt 1980; Lampert 1987; DeMott 1989). Of them, morphology and toxins have attracted the most interest (Lampert 1982; Fulton and Paerl 1987a; Lürling 2003a). However, distinguishing among these alternative determinants of food quality is not a simple matter. Many food-quality studies have been limited to simple comparisons of zooplankton performance on diets containing or lacking cyanobacteria (Arnold 1971; Porter and Orcutt 1980), a design that does not provide clear evidence for the role of alternative determinants given the large variety of physiological and morphological differences between cyanobacteria and other phytoplankters.

Large cyanobacterial forms, such as colonies and filaments, have been shown to mechanically interfere with zooplankton grazing by reducing ingestion of food particles (Webster and Peters 1978; Lynch 1980). An unequivocal test of the role of morphology would involve comparing zooplankton performance on diets consisting of the same strain of cyanobacteria but with contrasting morphologies. Such tests have been accomplished in relatively few studies in which a colonial or filamentous species has been disrupted into single cells or small clusters of cells via shaking or sonication. Some studies show that grazers perform better on larger forms of cyanobacteria (Chan et al. 2004), while other studies show that smaller forms of cyanobacteria are better foods for grazers than larger cyanobacteria (Hartmann 1985). These conflicting observations suggest that other factors, such as intracellular toxins and nutrient deficiencies, may interact with cyanobacterial morphology and/or grazer size in affecting zooplankton performance (Lampert 1987).

Unequivocal tests of the role of secondary metabolites are more difficult in practice and consequently rarer still. Cyanobacterial toxins have been hypothesized to be important determinants of the poor quality of cyanobacterial diets, but most studies have relied on additions of dissolved cyanobacterial extracts or purified toxins to zooplankton media (DeMott et al. 1991; Jungmann 1992), comparisons between a cyanobacterium and a starvation control (Lampert 1981a), comparisons between a nutritious alga and a cyanobacterium (Lampert 1981b), or comparisons between two strains of the same species of a cyanobacterium that differ in the production of a well-studied toxin (Smith and Gilbert 1995). In general, these types of comparisons do not provide unequivocal tests of the specific role of cyanobacterial toxins in driving poor zooplankton performance. In the above examples, the treatment diets compared may have differed in many ways besides the presence of a particular toxin (e.g., nutrient content, size, morphology). Studies employing a starvation control are limited to comparisons of survival rates and so do not fully address effects on population growth rate. In addition, tests of the effects of dissolved toxins do not mimic the typical route of exposure for freshwater grazers (via feeding) and have involved toxin exposure levels that are orders of magnitude higher than concentrations typically observed in freshwater lakes (DeMott et al. 1991; Chorus and Bartram 1999). Recent studies (Rohrlack et al. 1999a; Kaebernick et al. 2001; Lürling 2003a,b) comparing toxic, wild-type (contains several microcystin variants) and non-toxic, mutant (unable to synthesize microcystins) genotypes of the same cyanobacterial strain have so far provided less than definitive support for the importance of the most commonly-studied class of cyanobacterial toxin, microcystin, as a driver of poor zooplankton performance. For example, Lürling (2003)

reported similar population growth rates for grazers fed a toxic, wild-type strain of *Microcystis* and its non-toxic mutant.

In lieu of many direct tests of the effects of cyanobacterial morphology and toxicity on zooplankton performance, we conducted a meta-analysis of the dietary effects of cyanobacteria on zooplankton population growth rates to quantitatively synthesize the current state of knowledge. In this analysis, we compared population growth rates for zooplankton fed treatment foods containing cyanobacteria versus control foods comprising chlorophytes and/or flagellates. We also recognized that some experiments address the issue of cyanobacterial toxicity in a way (e.g., survivorship studies) that precludes them from being included in the above meta-analysis. For these studies, we conducted a supplementary meta-analysis to summarize experiments in which the survival of grazers fed toxic or non-toxic cyanobacteria was compared against a no-food (starvation) control. Comparison of a cyanobacterial diet against starvation is a long-standing approach to the assessment of dietary toxicity (Lampert 1981a), since it is assumed that grazers will die sooner in the presence of toxic food than when given no food at all. Such an experimental design might be open to alternative interpretation if grazers reduce their grazing effort in the absence of food (Plath 1998) and thus have higher survival when starved than when in the presence of nutritionally deficient cyanobacteria.

Given that a wide variety of cyanobacterial taxa with different morphologies and toxicological characteristics have been employed over a large number of experiments, meta-analysis provides a powerful means of quantitatively synthesizing this literature (Osenberg et al. 1999) and indirectly assessing the role of these alternative determinants

of food quality. We hypothesized that if morphology and/or toxin production are important determinants of the poor food quality of cyanobacteria in general, obvious differences in zooplankton performance should be apparent across these categories in the literature as a whole. Too few studies provided detailed information about nutritional composition to enable inclusion of nutritional inadequacy as a determinant of food quality in our analyses. We chose to focus on population growth rate (r) in assessing zooplankton performance, despite a plethora of performance measures employed in the literature, because population growth rate integrates over many alternative measures (e.g., feeding, assimilation, somatic growth, survival, reproduction), determines evolutionary fitness, and provides the most direct link to a major goal of much of this research, namely to understand zooplankton population and community dynamics as they are affected by cyanobacteria.

Methods

We searched the literature for experimental studies of the effect of cyanobacteria on zooplankton performance using Web of Science (from 1945 to 2004), Aquatic Sciences and Fisheries Abstracts (from 1971 to 2004), the reference lists of collected papers, and suggestions by scientists familiar with these types of studies. We included all comparisons that provided data on, or from which could be calculated, population growth rate for freshwater zooplankton fed a control diet (i.e., small chlorophyte and/or flagellated algae) versus a diet containing cyanobacteria (the "treatment" diet), or for a treatment diet containing toxic or non-toxic cyanobacteria versus no food (the "starvation" diet).

The primary analysis contrasted herbivore population growth rates on treatment versus control diets, as this was our main interest and many studies employed this design. No papers were found that measured population growth rate for copepods fed cyanobacteria-containing foods and a control food, thus only cladocerans and rotifers were included in the analysis. The 43 papers included in the primary meta-analysis comprised 358 experimental comparisons (Tables 1.1 and 1.3) across two or more dietary treatments including 19 cladoceran and 8 rotifer species, from 8 or 5 genera, respectively (Table 1.3). Twenty-two comparisons from seven studies were excluded from the data set (Table 1.4) because treatment *r* estimates could not be calculated, or control *r* estimates were near or below 0, which cannot be accommodated by our metric of effect size.

Table 1.1. List of studies used in the primary meta-analysis comparing *r* for grazers fed control foods comprised of chlorophytes and/or flagellates and treatment foods containing cyanobacteria. Grazer types: cl = cladoceran [Genera = [1]*Bosmina*, [2]*Ceriodaphnia*, [3]*Chydoris*, [4]*Daphnia*, [5]*Moina*, [6]*Moinadaphnia*, [7]*Scapholeberis*, [8]*Simocephalus*] and r = rotifer [Genera = [9]*Asplanchna*, [10]*Brachionus*, [11]*Hexarthra*, [12]*Keratella*, [13]*Synchaeta*]. Toxins analyzed and found to be present: [14]anatoxin, [15]deazaadenosine, [16]glucopyranoside, [17]microcystin. Morphology types: colonial = colonial [Genera = [18]*Microcystis*], filament = filamentous [Genera = [19]*Anabaena*, [20]*Aphanizomenon*, [21]*Cylindrospermopsis*, [22]*Oscillatoria*/*Planktothrix*], and single = single-celled [Genera = [19]*Anabaena*, [23]*Anacystis*, [24]*Gleocapsa*, [25]*Merismopedia*, [18]*Microcystis*, [26]*Synechococcus*, [27]*Synechocystis*]. ND = toxin presence not-determined by chemical analysis. * = studies employing toxic *Microcystis* PCC7820.

Studies	Grazer types	Cyanobacterial toxicity	Cyanobacterial morphology	Unweighted estimates	Weighted estimates
Ahlgren et al. 1990	cl[3,4]	ND	single[18], filamentous[22]	4	0
Alva-Martínez et al. 2001	cl[2,4,5]	ND	single[18]	9	9
Alva-Martínez et al. 2004	cl[4]	ND	single[18]	8	8
Arnold 1971	cl[4]	non-toxic, ND	single[19,23,24,25,26,27]	14	0
Brett 1993	cl[4]	ND	single[18]	2	2
Cecchine 1997	r[10]	toxic[17]	single[18]	9	9
Chen and Xie 2003	cl[5]	ND	colonial[18]	3	0
Chen and Xie 2004*	cl[4]	toxic[17]	single[18]	4	0
Claska and Gilbert 1998	cl[4]	toxic[14,15,16]	filament[19]	25	23
de Bernardi et al. 1981	cl[4]	ND	single[18]	6	0
Ferrão-Filho and Azevedo 2003	cl[2,5]	toxic[17]	single[18], colonial[18]	16	16
Ferrão-Filho et al. 2000	cl[2,4,5,6]	toxic[17]	single[18]	25	23
Ferrão-Filho et al. 2002	cl[2,5]	toxic[17]	single[18]	2	2
Fulton and Paerl 1987b	r[10]	ND	single[18], colonial[18]	4	0
Fulton and Paerl 1988	cl[4], r[10]	ND	colonial[18]	4	0
Gilbert 1990	cl[1,2,4], r[12,13]	toxic[15,16]	filament[19]	22	22
Gilbert 1994	r[12]	toxic[14]	filament[19]	1	0
Gilbert 1996*a*	r[10]	toxic[14]	filament[19]	5	4
Gilbert 1996*b*	r[9,10]	toxic[14]	filament[19]	6	6
Gilbert and Durand 1990	cl[4], r[12]	non-toxic	filament[19]	4	4
Hanazato and Yasuno 1987	cl[5]	ND	single[18], colonial[18]	2	0
Henning et al. 1991	cl[4]	toxic[17], non-toxic, ND	single[18]	4	0
Hietala et al. 1995*	cl[4]	toxic[17]	single[18]	10	8
Hietala et al. 1997*a**	cl[4]	toxic[17]	single[18]	18	15
Hietala et al. 1997*b**	cl[4]	toxic[17]	single[18]	4	4
Kurmayer 2001	cl[1,4]	non-toxic	filament[20]	4	4
Lundstedt and Brett 1991	cl[4]	ND	single[18]	1	0
Lürling 2003*a*	cl[4]	toxic[17], non-toxic	single[18]	8	8
Lürling 2003*b**	cl[4]	toxic[17], non-toxic	single[18]	16	16
Lürling and van der Grinten 2003	cl[4]	toxic[17], non-toxic	single[18]	5	5
Nandini and Rao 1998	cl[2,4,5,7,8], r[10,11]	ND	single[18], colonial[18]	32	32
Nandini et al. 2000	cl[4]	ND	colonial[18]	2	2
Porter and Orcutt 1980	cl[4]	toxic[14]	single[19], filament[19]	4	0
Repka 1996	cl[4]	toxic[17]	filament[22]	6	0
Repka 1997	cl[4]	toxic[17]	filament[22]	9	9
Repka 1998	cl[4]	toxic[17]	filament[22]	12	12
Rothhaupt 1991	r[10]	ND	single[18], filament[19,21]	8	5
Sartonov 1995	cl[4], r[12]	non-toxic	single[18]	4	4
Shurin and Dodson 1997	cl[4]	toxic[17]	single[18]	3	3
Smith and Gilbert 1995*	cl[4], r[12]	toxic[17], non-toxic	single[18]	18	17
Starkweather 1981	r[10]	non-toxic	filament[19]	2	0
Starkweather and Kellar 1983	r[10]	toxic[14]	filament[19]	3	3
Weithoff and Walz 1995	r[10]	toxic[17]	filament[22]	10	10
			Total	358	285

Table 1.2. List of studies used in secondary meta-analysis comparing *r* derived from survivorship curves, LT_{50} estimates, or directly from papers for grazers fed treatment foods containing cyanobacteria and no food. Data types: survivor = survivorship curves, LT_{50} = LT_{50} estimates, and *r* = *r* provided in or calculated from paper. Grazer types: ana = anostracan [Genus = [1]*Thamnocephalus*], cl = cladoceran [Genera = [2]*Bosmina*, [3]*Ceriodaphnia*, [4]*Daphnia*, [5]*Moina*, [6]*Moinadaphnia*], cop = copepod [Genera = [7]*Cyclops*, [8]*Diaptomus*, [9]*Eudiaptomus*], and r = rotifer [Genus = [10]*Brachionus*]. Toxins analyzed and found to be present: [11]anatoxin, [12]cylindrospermopsin, [13]deazaadenosine, [14]glucopyranoside, [15]microcystin, [16]microviridin, [17]paralytic shellfish toxin]. Morphology types: filament = filamentous [Genera = [18]*Anabaena*, [19]*Aphanizomenon*, [20]*Cylindrospermopsis*], and single = single-celled [Genera = [18]*Anabaena*, [21]*Microcystis*, [22]*Planktothrix*]. ND = toxin presence not-determined by chemical analysis. * = studies employing toxic *Microcystis* PCC7820.

Studies	Data type	Grazer types	Cyanobacterial toxicity	Cyanobacterial morphology	Treatment food - no food
Cecchine 1997	*r*	r[10]	toxic[15]	single[21]	1
DeMott et al. 1991*	survivor	cl[4], cop[8]	toxic[15], non-toxic	single[21]	12
Ferrão-Filho et al. 2000*	survivor	cl[3,4,5,6]	toxic[15]	single[21]	97
Fulton 1988*	survivor	cl[2,4,5]	toxic[11,15]	single[21], filament[18]	8
Gilbert 1998	survivor	cl[4]	toxic[13,14]	filament[18]	2
Henning et al. 1991	*r*	cl[4]	toxic[15], non-toxic	single[21]	2
Henning et al. 2001	survivor	cl[4]	toxic[15], non-toxic	single[21]	5
Kaebernick et al. 2001	survivor	cl[4]	toxic[15], non-toxic	single[21]	4
Kurmayer and Jüttner 1999	survivor	ana[1], cl[4], cop[7,9]	toxic[15]	single[22]	4
Lürling 2003*a*	*r*	cl[4]	toxic[15], non-toxic	single[21]	8
Matveev et al. 1994	survivor	cl[4]	toxic[15]	single[21]	1
Nizan et al. 1986*	survivor	cl[4]	toxic[15]	single[21]	4
Nogueira et al. 2004*a*	survivor	cl[4]	toxic[12], non-toxic	filament[20]	2
Nogueira et al. 2004*b*	survivor	cl[4]	toxic[17]	filament[19]	1
Porter and Orcutt 1980	*r*	cl[4]	toxic[11]	single[18], filament[18]	4
Reinikainen et al. 1994*	survivor	cl[4]	toxic[15]	single[21]	48
Rohrlack et al. 1999*a*	survivor	cl[4]	toxic[15], non-toxic	single[21]	2
Rohrlack et al. 1999*b*	LT_{50}	cl[4]	toxic[15], non-toxic	single[21]	6
Rohrlack et al. 2001a	LT_{50}	cl[4]	toxic[15], non-toxic	single[21]	12
Rohrlack et al. 2004	survivor	cl[4]	toxic[16]	single[21]	2
Smith and Gilbert 1995	*r*	cl[4]	non-toxic	single[21]	1
Thostrup and Christoffersen 1999	survivor	cl[4]	toxic[15]	single[21]	1
van der Grinten et al. 2000	survivor	cl[4]	toxic[15], non-toxic	single[21]	12
				Total	239

Table 1.3. Grazer genera included in the primary and secondary meta-analyses comparing *r* for grazers fed control foods comprised of chlorophytes and/or flagellates and treatment diets containing cyanobacteria.

Meta-analysis	Genera	Grazer group	Number of species	Unweighted effect size estimates
primary	*Asplanchna*	rotifer	1	3
primary	*Bosmina*	cladoceran	1	3
primary	*Brachionus*	rotifer	2	50
primary	*Ceriodaphnia*	cladoceran	2	34
primary	*Chydoris*	cladoceran	1	2
primary	*Daphnia*	cladoceran	10	200
primary	*Hexarthra*	rotifer	1	4
primary	*Keratella*	rotifer	3	19
primary	*Moina*	cladoceran	2	27
primary	*Moinadaphnia*	cladoceran	1	6
primary	*Scapholeberis*	cladoceran	1	4
primary	*Simocephalus*	cladoceran	1	4
primary	*Synchaeta*	rotifer	1	2
primary		Total	27	358
secondary	*Bosmina*	cladoceran	1	2
secondary	*Brachionus*	rotifer	1	1
secondary	*Ceriodaphnia*	cladoceran	1	16
secondary	*Cyclops*	copepod	1	1
secondary	*Daphnia*	cladoceran	8	164
secondary	*Diaptomus*	copepod	1	4
secondary	*Eudiaptomus*	copepod	1	1
secondary	*Moina*	cladoceran	1	21
secondary	*Moinadaphnia*	cladoceran	1	28
secondary	*Thamnocephalus*	anostracan	1	1
secondary		Total	17	239

Table 1.4. List of excluded comparisons not used in the primary and secondary meta-analyses.

Meta-analysis	Studies	Comparisons excluded	Reason for exclusion
primary	Ahlgren et al. 1990	2	control *r* near or below 0
primary	Chen and Xie 2003	9	control *r* near or below 0
primary	Ferrão-Filho et al. 2000	4	unable to calculate treatment *r*
primary	Laurén-Määttä et al. 1997	4	control *r* near or below 0
primary	Lundstedt and Brett 1991	2	control *r* near or below 0
primary	Weithoff and Walz 1995	1	control *r* near or below 0
primary	Total	22	
secondary	Arnold 1971	14	starvation *r* above 0
secondary	Total	14	

In most cases (99%), zooplankton population growth rates for grazers fed control foods were reported in the papers used in the primary analysis. In the few cases where control *r* values were not reported (Lundstedt and Brett 1991; Gilbert 1994), we calculated *r* from initial and final population densities ($r = \frac{\ln density_{t+1} - \ln density_t}{time}$). Most (90%) population growth rate estimates for grazers fed treatment foods also came directly from the studies, however a few studies did not provide estimates of *r* (Fig. 1.1), presumably due to lack of reproduction or because all of the experimental animals died during the assays. We did not want to exclude these studies since they provided worst-case scenarios (i.e., large negative effects of cyanobacteria) for experimental comparisons, so we estimated *r* for these studies from a lack of reproduction but no mortality ($r = 0$; $n = 4$) and survivorship curves ($n = 33$)**.** In those cases where all grazers died prior to the end of the experiment, we used a population size of 1 on the day the last grazer died to calculate *r*.

We included all comparisons regardless of whether estimates of among-replicate variance were provided and conducted an unweighted meta-analysis, to avoid biasing the data set. Employing data-exclusion criteria that are not specifically tied to the data's relevance to the question or that relate only to study "quality", can lead to spurious conclusions (Englund et al. 1999). However, given that variance-weighted estimates enable more powerful statistical tests, we also extracted within-replicate variances, when available, for use in a parallel set of variance-weighted meta-analyses. We compared the two sets of analyses to assess the robustness of the results (Englund et al. 1999).

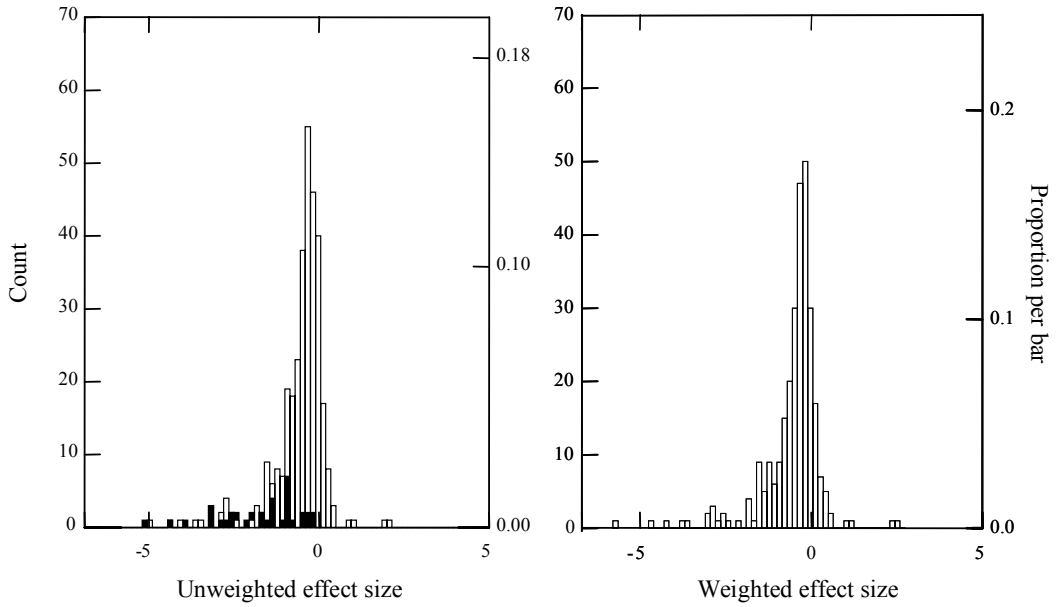

Figure 1.1. Frequency distribution of effect sizes for unweighted and weighted estimates of *r* comparisons for grazers fed control foods comprised of chlorophytes and/or flagellates and treatment foods containing cyanobacteria used in the primary meta-analysis. Most effect sizes were calculated using *r* estimates collected directly from the studies (white bars), however, a few *r* estimates were calculated from survivorship data (black bars).

Additional information from each study was recorded to enable categorization of the data by cyanobacterial morphology (single-celled, colonial, filamentous), cyanobacterial toxicity (non-toxic, toxic, not determined), zooplankton type (cladocerans, rotifers), adult zooplankton length (actual lengths provided in most studies, otherwise estimates were collected from the literature), control food type, carbon concentration of control and treatment foods (provided directly in many studies, otherwise carbon concentration was estimated by calculating biovolume from cell density and cell dimensions [actual measurements provided in most studies, otherwise estimates were collected from the literature] and assuming; a specific gravity of 1, a dry mass:wet mass ratio of 0.10, and a carbon content of dry biomass of 50% (Riemann et al. 1989)), and percent cyanobacterial carbon in treatment foods.

The classification of a cyanobacterium as toxic or non-toxic is central to our analysis but not straightforward. In vitro assays (e.g., ELISA, HPLC, LC/MS) for chemically-described secondary metabolites having an established toxicity toward some organism(s) may miss inhibitory compounds that have yet to be described. Relying on such assays for classification might result in some toxic cyanobacteria being misclassified as non-toxic. Alternatively, bioassays employing terrestrial animals or aquatic animals with no previous evolutionary history of exposure to the compound may result in a compound being characterized as toxic when it is not inhibitory to animals that coexist with the compound in nature. In this case, non-toxic strains would be misclassified as toxic. These ambiguities cannot be resolved by any classification scheme. Indeed, one of the main motivations for this work was to assess whether well-known compounds are indeed inhibitory, in a general sense, to zooplankton population

growth when part of the diet. Consequently, we chose to focus on the following specific question: Does the presence or absence of previously-described secondary metabolites with toxic effects on some organism have an influence on the population growth rate of zooplankton, in general?

Cyanobacteria were considered toxic if they tested positive via in vitro analysis for known toxins (anatoxin, cylindrospermopsin, deazaadenosine, glucopyranoside, microcystin, microviridin, and paralytic shellfish toxin). Toxin concentrations are known to vary within and among cyanobacterial strains under different environmental conditions and physiological states (Lee et al. 2000), however these data were not routinely provided and consequently could not be included in our analysis. Non-toxic cyanobacteria were those that either tested negative for known toxins via in vitro analysis or in one case (Arnold 1971) were considered non-toxic because the strains were members of genera that, to our knowledge, have never been shown to produce toxins (i.e., *Gleocapsa*, *Merismopedia*, *Synechocystis*). Cases in which potentially toxic genera of cyanobacteria (e.g., *Microcystis*, *Anabaena, Oscillatoria/Planktothrix*) were not chemically analyzed for toxins were designated as "not determined". We contacted authors to obtain information about the toxicity of cyanobacterial strains when this information was not included in the original papers. This inquiry netted an additional 45 comparisons that could be classified by toxicity for a total of 265 non-toxic versus toxic comparisons for the primary analysis.

For the primary analysis, the effect of cyanobacteria on zooplankton population growth relative to control foods was calculated for each comparison (n = 358) as:

$$\textit{Unweighted effect size} = \frac{r_t - r_c}{r_c}$$

where r_t is the population growth rate on the cyanobacterial (treatment) diet and r_c is the population growth rate on the control diet (e.g., small chlorophyte and/or flagellate). This metric was chosen to standardize effect sizes by maximum population growth rate (i.e., growth rate on control foods) across taxa with varying growth rates and center the results about zero to facilitate interpretation. An effect size of zero indicates that growth rates on the two diets were the same. Negative effect sizes indicate that growth rates were lower on the cyanobacterial diet, i.e., that zooplankton growth was inhibited by cyanobacteria relative to a control diet. Effect sizes less than -1 indicate that population growth on the cyanobacterial diet (r_t) was negative since r_c was always greater than zero. Weighted effect sizes (n = 285) were calculated as:

$$\textit{Weighted effect size} = \frac{r_t}{r_c} + \frac{r_t \times \operatorname{var} r_c}{{r_c}^3} - 1$$

Variances for these weighted effects sizes were calculated as:

$$\textit{Weighted variance} = \frac{\operatorname{var} r_t}{{r_c}^2} + \frac{\operatorname{var} r_c}{{r_c}^4}$$

In the secondary meta-analysis, 23 papers provided data (n = 239) for cladocerans (5 genera and 12 species), copepods (3 genera and 3 species), a rotifer (1 genus and 1 species), and a fairy shrimp (1 genus and 1 species) fed non-toxic (n = 27) or toxic (n = 212) cyanobacteria versus no food (Tables 1.2 and 1.3). Data for one study (Arnold 1971) were not included because starved grazers exhibited positive population growth, suggesting a methodological problem in the starvation treatment (Table 1.4). In this analysis, estimates of r (measured as survival rate) were collected directly or calculated from initial and final densities. In those cases where all grazers died prior to the end of

the experiment, we used a population size of 1 on the day the last grazer died to calculate r.

A different effect size metric was needed for the secondary analysis since the data in most of these studies were limited to survivorship estimates over time, which result in population growth rate estimates ≤ 0. Estimates of $r \leq 0$ cannot be accommodated by the effect size metric above ($\frac{r_t - r_c}{r_c}$), so we calculated effect sizes for grazers fed cyanobacteria-containing foods (treatment) versus starved grazers as:

$$\textit{Difference effect size metric} = r_t - r_s$$

where r_t is the survival rate on the cyanobacterial (treatment) diet and r_s is the survival rate for starved grazers. An effect size of zero indicates that survival rates on the two diets were the same. Negative effect sizes indicate that survival rates were lower on the cyanobacterial diet, i.e., that zooplankton survival was inhibited by cyanobacteria relative to starvation.

Population growth rates for grazers fed control or treatment foods were compared using a paired *t*-test. Analysis of variance (ANOVA) and its weighted analog (MetaWin v. 2.1) were used to test for differences in effect size among categorical means and to identify significant interactions, followed by Tukey's multiple comparison tests when more than two means were being compared. We used random-effects models for all categorical-weighted analyses because we did not assume a single, true effect size across the disparate studies, as is done for fixed-effects models. Linear regression was used to examine relationships between effect sizes and grazer size or percent cyanobacteria in treatment foods. A general linear model (GLM) was used to test for interactions (i.e., homogeneity of slopes test) between grazer size and cyanobacterial morphology or

toxicity. Analysis of covariance (ANCOVA) was used to test for effects of cyanobacterial toxicity and morphology after accounting for variation attributable to continuous variables (i.e., grazer size, percent cyanobacteria in the treatment diet). ANCOVA was employed only if slopes (e.g., effect size vs. grazer length or percent cyanobacteria in the treatment diet) were not significantly different at $p < 0.05$. Unweighted and weighted analyses were performed with Systat 9.01 (SPSS 1998) and MetaWin v. 2.1 (Rosenberg et al. 2002), respectively. The rejection criterion was set a priori at $\alpha < 0.05$.

Results

As expected, zooplankton population growth rates were much lower (paired *t*-test $p < 0.001$) on diets containing cyanobacteria (0.191 ± 0.024; mean ± SE) than on control diets (0.479 ± 0.025; mean ± SE), and as a result, mean effect sizes averaged over all studies were negative (Table 1.5, Fig. 1.3, mean effect sizes significantly < 0 at $p < 0.001$, *t*-test). The magnitude of growth inhibition by cyanobacteria also increased with the cyanobacterial fraction of the treatment diet (Table 1.6, Fig. 1.4a; $p < 0.001$). Significant inhibition of zooplankton population growth was evident for all three morphological types (effect sizes for all morphological types significantly < 0 at $p < 0.001$, *t*-tests), but single-celled and colonial forms inhibited growth more than filamentous forms overall (Table 1.5, Fig. 1.3). Surprisingly, there was no statistically significant difference in effect size for toxic versus non-toxic cyanobacteria despite large sample sizes (total $n = 265$) and low residual error (Table 1.5, Fig. 1.3; $p = 0.446$).

We also examined interactions between grazer length, morphology, and toxicity and cyanobacterial morphology and toxicity. Our analysis of the interaction between morphology and toxicity was limited by the complete lack of non-toxic colonial species in the data set, and so only includes filamentous versus single-celled morphologies. No significant interactions were found between cyanobacterial toxicity and grazer length (toxicity $p = 0.993$, grazer length $p = 0.001$, toxicity x grazer length interaction $p = 0.248$) or cyanobacterial morphology and toxicity (morphology $p < 0.001$, toxicity $p = 0.020$, morphology x toxicity interaction $p = 0.313$) for all grazers. However, a significant interaction was found between cyanobacterial morphology and grazer length (morphology $p < 0.001$, grazer length $p = 0.035$, morphology x grazer length interaction

$p = 0.004$), Moreover, the effect of cyanobacteria across grazer lengths varied among cyanobacterial types (Table 1.6). Effect sizes were negatively related to increasing grazer length for filamentous, non-toxic, and toxic cyanobacteria (Table 1.6, $p \leq 0.019$), positively related to increasing grazer length for colonial cyanobacteria (Table 1.6, $p = 0.036$), but no relationship was found between grazer length and single-celled cyanobacteria (Table 1.6, $p = 0.667$).

There was no statistically significant difference in inhibition between cladocerans and rotifers over all studies (Fig. 1.3; $p = 0.110$), which is consistent with the lack of a significant relationship between effect size and grazer length across all studies (Table 1.6, Fig. 1.4b; $p = 0.428$). Moreover, neither type of grazer responded differently to toxic versus non-toxic cyanobacteria (Table 1.5, Fig. 1.3; $p \geq 0.49$). However, when analyzed separately, cladocerans and rotifers did respond somewhat differently to the distinct cyanobacterial morphologies (Table 1.5, Fig. 1.3; $p \leq 0.002$). Both grazer groups were inhibited more by single-celled cyanobacteria than filamentous cyanobacteria, but rotifers were more negatively affected by colonial and single-celled cyanobacteria than cladocerans (Fig. 1.3; $p \leq 0.005$). Interestingly, rotifer performance on filamentous cyanobacteria was similar to that on control foods (Table 1.5, Fig. 1.3), however the mean effect size was negative and nearly significantly less than zero (95% CI bounds 0; *t*-test mean = 0, $p = 0.056$). Unweighted and weighted analyses provided similar outcomes in most of the above cases (Tables 1.5 and 1.6).

Given the unexpected and potentially controversial finding that cyanobacterial toxicity had little influence on mean effect size in studies comparing cyanobacteria to a control diet, we analyzed the primary data set more intensively to examine the robustness

of this specific result. First, we examined whether there was an interaction between the concentration of cyanobacteria in treatment foods and presence or absence of toxicity. There was no interaction between cyanobacterial concentration and toxicity ($p = 0.477$) and no statistically significant effect of toxicity after accounting for variation in cyanobacterial concentration (ANCOVA; percent cyanobacteria $p < 0.001$, toxicity $p = 0.378$). Second, we restricted the data set to studies in which the treatment diet was made up of 100% cyanobacteria given that these treatments showed the strongest negative effects on population growth (Fig. 1.4a). Again, effect sizes between toxic and non-toxic cyanobacteria were not significantly different for neither all grazers ($p = 0.354$), nor for cladocerans ($p = 0.527$) and rotifers ($p = 0.355$) analyzed separately. Finally, we examined the influence of variation in the control diet on effect size. Zooplankton exhibited greater population growth on control diets made up of *Cryptomonas*, *Nannochloris*, or *Euglena* relative to other species (overall $p < 0.001$; Fig. 1.2). We analyzed the data after subdividing effect sizes into two categories based on which type of control food was employed (better food: *Cryptomonas*, *Euglena*, and *Nannochloris*; poorer food examples: *Chlorella* and *Scenedesmus*). Neither data subset revealed statistically significant differences in mean effect size for grazers fed toxic or non-toxic cyanobacteria ($p \geq 0.473$).

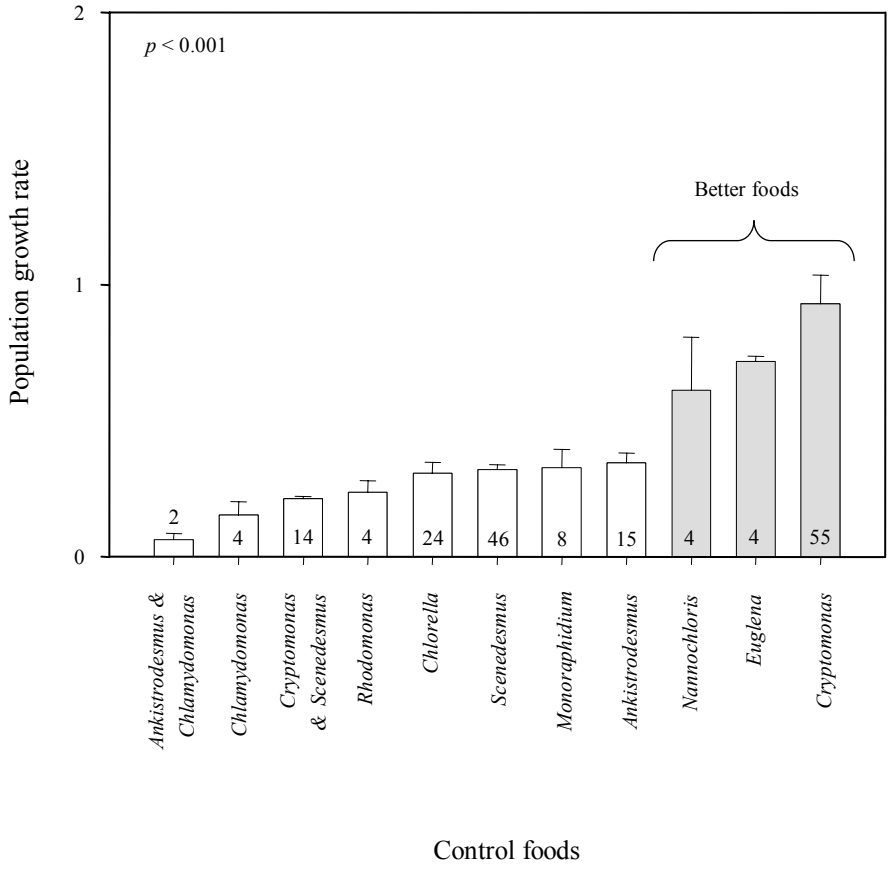

Figure 1.2. Control food population growth rates (mean ± 1 SE) for grazers used in the primary meta-analysis. Inset numbers indicate sample size for each estimate where each control food growth rate estimate was used only once per study, even if multiple effect size estimates incorporated the same control food growth rate estimate more than once.

Table 1.5. Results for the primary meta-analysis comparing *r* for grazers fed control foods comprised of chlorophytes and/or flagellates and treatment foods containing cyanobacteria. *n* = sample size. CI = confidence interval. CIs containing zero signify that the treatment and control foods affected grazers equally. *p*-values relate to specific effect size comparisons (e.g., cladocerans versus rotifers, non-toxic versus toxic cyanobacteria). Differences in letter (^{a,b}) indicate significant differences as detected by Tukey's multiple comparison tests.

Comparisons	Groups	Unweighted *n*	Unweighted mean effect size	Unweighted 95% CI	Unweighted *p*-values	Weighted *n*	Weighted mean effect size	Weighted 95% CI	Weighted *p*-values
Overall effect		358	-0.673	-0.768 to -0.579		285	-0.412	-0.450 to -0.374	
Toxicity	non-toxic	45	-0.457	-0.606 to -0.308	0.446	34	-0.368	-0.463 to -0.272	0.355
	toxic	220	-0.544	-0.642 to -0.447		193	-0.320	-0.360 to -0.281	
Morphology	single-celled	199	-0.836[a]	-0.967 to -0.704	0.000	155	-0.612	-0.669 to -0.556	0.001
	colonial	38	-0.905[a]	-1.309 to -0.501		28	-0.568	-0.735 to -0.400	
	filamentous	119	-0.325[b]	-0.436 to -0.215		101	-0.252	-0.298 to -0.207	
Grazer type	cladocerans	280	-0.633	-0.723 to -0.542	0.110	220	-0.386	-0.432 to -0.340	0.038
	rotifers	78	-0.819	-1.109 to -0.529		65	-0.473	-0.542 to -0.403	
Cladocerans only									
Toxicity	non-toxic	32	-0.513	-0.712 to -0.315	0.494	23	-0.401	-0.517 to -0.285	0.280
	toxic	177	-0.600	-0.700 to -0.500		152	-0.335	-0.382 to -0.288	
Morphology	single-celled	170	-0.747[a]	-0.866 to -0.629	0.002	130	-0.599	-0.658 to -0.541	0.000
	colonial	30	-0.627[a,b]	-1.026 to -0.228		24	-0.372	-0.560 to -0.184	
	filamentous	78	-0.380[b]	-0.502 to -0.258		66	-0.195	-0.241 to -0.150	
Rotifers only									
Toxicity	non-toxic	13	-0.318	-0.500 to -0.136	0.994	11	-0.292	-0.490 to -0.093	0.951
	toxic	43	-0.316	-0.598 to -0.033		41	-0.286	-0.366 to -0.205	
Morphology	single-celled	29	-1.354[a]	-1.921 to -0.786	0.000	25	-0.683	-0.845 to -0.522	0.000
	colonial	8	-1.947[a]	-3.026 to -0.868		4	-1.420	-2.086 to -0.755	
	filamentous	41	-0.221[b]	-0.447 to 0.006		36	-0.336	-0.459 to -0.213	

Table 1.6. Linear regression output for the unweighted and weighted analyses of the primary data set comparing *r* for grazers fed control foods comprised of chlorophytes and/or flagellates and treatment foods containing cyanobacteria. *n* = sample size.

Independent variable	Dependent variable	Unweighted *n*	Unweighted slopes	Unweighted *p*-values	Weighted *n*	Weighted slopes	Weighted *p*-values
Percent	all grazers	358	-0.008	0.000	285	-0.005	0.000
cyanobacterial	cladocerans	280	-0.005	0.000	220	-0.003	0.000
carbon in	rotifers	78	-0.020	0.000	65	-0.013	0.000
treatment diet	non-toxic	45	-0.008	0.000	34	-0.011	0.000
(%)	toxic	220	-0.006	0.000	193	-0.002	0.000
	single-celled	199	-0.010	0.000	155	-0.012	0.000
	colonial	38	-0.017	0.022	28	-0.012	0.018
	filamentous	119	-0.005	0.000	102	-0.001	0.034
Grazer adult	all grazers	355	-0.033	0.428	282	-0.036	0.035
size (mm)	non-toxic	45	-0.151	0.003	34	-0.139	0.007
	toxic	217	-0.243	0.000	190	-0.092	0.000
	single-celled	199	0.024	0.667	155	-0.061	0.102
	colonial	38	0.605	0.036	28	0.412	0.060
	filamentous	116	-0.142	0.019	99	0.057	0.002

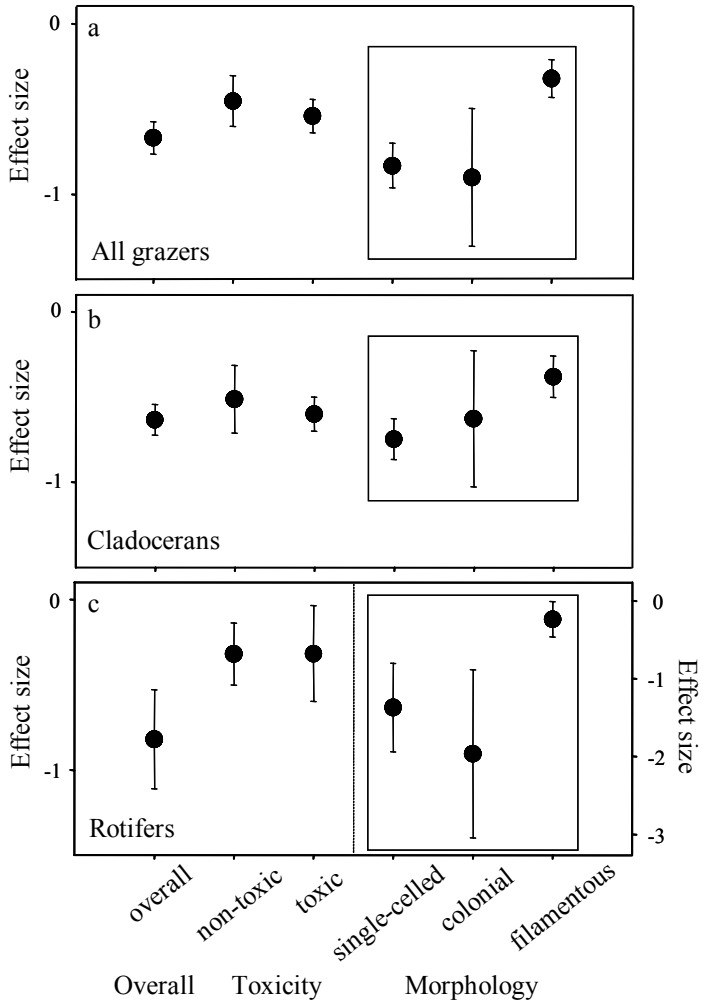

Figure 1.3. Effect size averages and 95% confidence intervals for the unweighted data set from the primary meta-analysis comparing *r* for (a) all grazers, (b) cladocerans only, and (c) rotifers only fed control foods composed of chlorophytes and/or flagellates and treatment foods containing cyanobacteria. A box enclosing mean effect sizes indicates that the values were significantly different from each other ($p < 0.05$). Note scale changes for cyanobacterial morphology mean effect sizes for rotifers. Sample sizes and statistical results presented in Table 1.5.

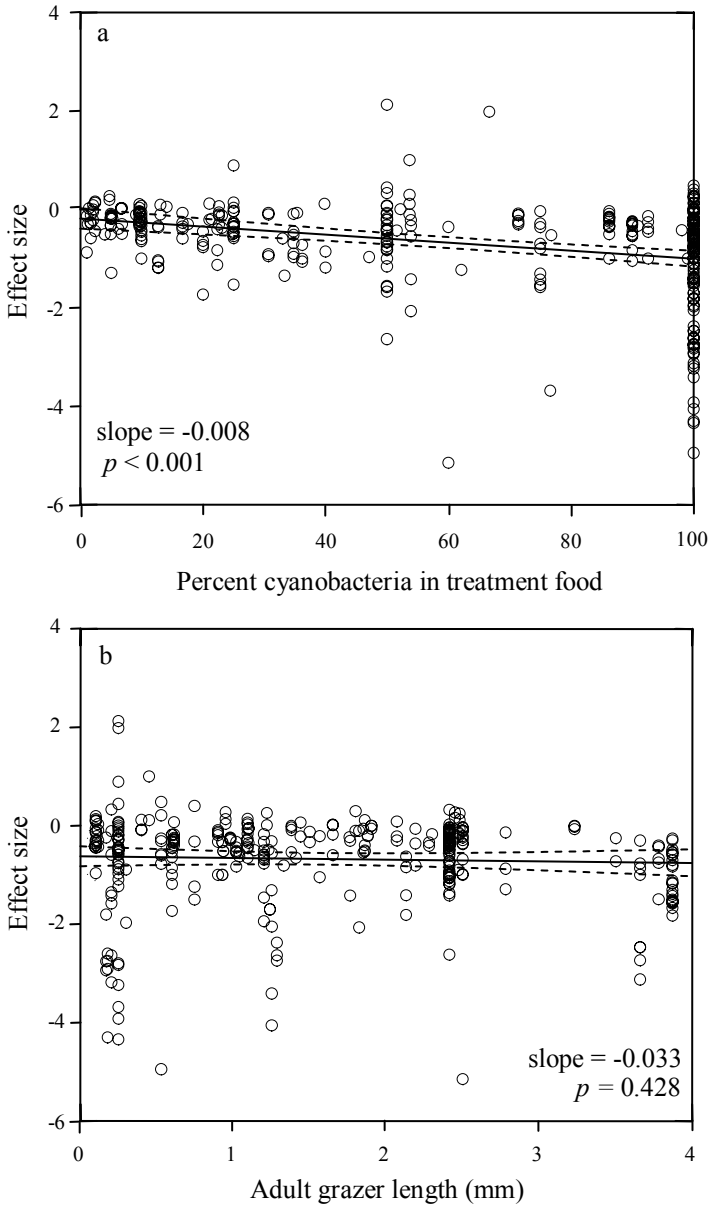

Figure 1.4. Relationships between unweighted effect size and (a) percent cyanobacterial concentration in treatment foods or (b) adult grazer length (mm) for the primary data set comparing *r* for grazers fed control foods comprised of chlorophytes and/or flagellates and treatment foods containing cyanobacteria. Solid lines are best-fit lines. Dashed lines are 95% confidence bands. Transformation to remove heteroscedasticity did not change the results of the regression appreciably.

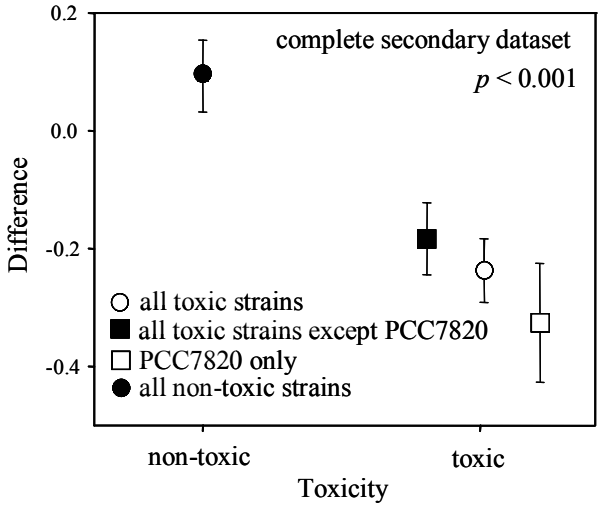

Figure 1.5. Effect size differences (metric: $r_t - r_s$ where r_t is the survival rate on the cyanobacterial (treatment) diet and r_s is the survival rate with no food) and 95% confidence intervals for the secondary analysis. Toxic cyanobacterial data presented as all toxic strains, all toxic cyanobacteria excluding PCC7820, and only data for PCC7820. Complete secondary analysis refers to all non-toxic strains versus all toxic strains.
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In direct contrast to the lack of influence of toxicity in the analysis of experiments employing control foods (Table 1.5, Fig. 1.3), the secondary analysis of experiments employing a starvation treatment revealed that toxic cyanobacteria (Fig. 1.5; mean = -0.237, SE = 0.027, $n = 212$) inhibited zooplankton more than non-toxic cyanobacteria (Fig. 1.5; mean = 0.093, SE = 0.030, $n = 27$; significantly different at $p < 0.001$). Grazers fed toxic cyanobacteria exhibited significantly lower survival than starved animals (*t*-test mean = 0, $p < 0.001$), whereas grazers fed non-toxic cyanobacteria exhibited significantly higher survival than starved animals (*t*-test mean = 0, $p = 0.005$). This data set was heavily biased towards the use of *Microcystis* (93% of the cyanobacteria treatment food types in the secondary analysis), and more specifically, the microcystin-containing strain *M. aeruginosa* PCC7820 (37% of the toxic treatment foods in the secondary meta-analysis, compared with only 21% of the toxic treatment foods in the larger, primary analysis). Repeated use of the same species or genotype is an obvious source of non-independence in meta-analysis (Gurevitch and Hedges 1999), and may limit the domain of relevance of our synthesis, in much the same way that pseudoreplication affects the interpretation of an individual experiment (Hurlbert 1984). It is possible that the significant influence of toxicity in the secondary analysis is driven solely by the effect of this single genotype of *M. aeruginosa*. To address this possibility, we examined the influence of toxicity for experiments using PCC7820 versus experiments using all other toxic strains (Fig. 1.5). This analysis revealed that the influence of toxicity on zooplankton survival rates is statistically significant even in the absence of PCC7820 (non-toxic results presented above; all other toxic strains besides PCC7820 mean = -0.184, SE = 0.031, $n = 133$, all other toxic strains vs. non-toxic $p < 0.001$; PCC7820 data

only mean = -0.326, SE = 0.051, n = 79, PCC7820 vs. non-toxic $p < 0.001$), but also that the magnitude of the difference in effect sizes between comparisons for toxic and non-toxic cyanobacteria is much less (44% less) for all other strains than for PCC7820 (Fig. 1.5).

Discussion

Using two meta-analyses of published data, we confirmed that cyanobacteria are generally poor food for freshwater grazers relative to small chlorophytes and/or flagellates, a generalization that is widely presumed to be true (Porter and Orcutt 1980; Sommer 1989; Gilbert 1990). The very low *p*-values associated with the general effect of cyanobacteria and for the effect of increasing concentrations of cyanobacteria ($p < 0.001$) indicate that our analysis was relatively powerful, as would be expected given the generally large samples sizes (Tables 1.1, 1.2., 1.3, 1.5, and 1.6). Despite these large sample sizes, however, we did not find an overall statistical difference in population growth inhibition between toxic and non-toxic cyanobacteria (as defined in our analysis), a generalization that is also widely presumed to be true. We also found that cladocerans and rotifers were affected differently by the distinct cyanobacterial morphologies, a finding that has not been widely recognized before (but see: Gilbert 1990; Nandini and Rao 1998).

In general, cladocerans and rotifers responded similarly to cyanobacteria regardless of toxin absence or presence, relative to control foods. These findings were somewhat unexpected given that cladocerans and rotifers use different chemo- and mechanosensory structures to locate and ingest prey (Larsson and Dodson 1993; Snell 1998). However, we did find statistically significant differences between the two grazer groups in their responses to different cyanobacterial morphologies (Fig. 1.3). Cladocerans and rotifers both grew best on filamentous forms, but rotifers were more inhibited by single-celled and colonial cyanobacteria whereas cladocerans were most

inhibited when fed single-celled cyanobacteria (Table 1.5, Fig. 1.3; Tukey's tests, $p < 0.05$).

Across 27 species of freshwater grazers incorporated in the primary analysis (Table 1.3), filamentous algae were a better food source than single-celled cyanobacteria when compared to grazers fed control foods. This finding is surprising given that filamentous cyanobacteria have been shown to mechanically interfere with herbivore filtering appendages (Lynch 1980; Porter and McDonough 1984; Gliwicz 1990), thus reducing ingestion of food particles. We consider two explanations for the difference between the effects of filamentous and single-celled cyanobacteria. First, for phylogenetic reasons, filamentous and single-celled genera may lack different essential nutrients. For example, the filamentous cyanobacterium *Anabaena* has been found deficient in sterols (von Elert et al. 2003), while a strain of single-celled *Synechococcus* lacked essential fatty acids (von Elert and Wolffrom 2001). Alternatively, single-celled cyanobacteria may be ingested more readily than filamentous algae due to grazer gape limitations (Gliwicz and Siedlar 1980), and these smaller prey may contain undescribed secondary metabolites that function as toxins only when consumed (Wolfe 2000).

Single-celled cyanobacteria occur in nature (e.g., *Chroococcus*, *Dactylococcopsis*, *Synechococcus, Synechocystis*), but we are unaware of any naturally-occurring, toxic, single-celled, freshwater cyanobacterial species. Given that *Microcystis*, a strictly colonial genus (Komárek 1991), composed 62% of the treatment foods types in our primary meta-analysis, our observation that relatively few studies measured population growth for grazers fed colonial cyanobacteria is surprising. However, *Microcystis* strains from culture collections, as near as we can ascertain, almost invariably grow as single

cells, and many (35%) of the papers in our primary meta-analysis utilized culture-collection strains of this genus. Eighty-three percent of the effect size comparisons involving *Microcystis* used single-celled strains. Given the importance of morphology as a determinant of food quality (Tables 1.5 and 1.6, Fig. 1.3) and grazing-resistance (Sterner 1989), widespread use of single-celled strains of colonial species in studies of phytoplankton-zooplankton interactions is problematic. This problem can be overcome by employing cyanobacteria that have been recently collected from the field (Nandini and Rao 1998) or remain colonial in culture (Ferrão-Filho and Azevedo 2003), where *Microcystis* can retain the colonial habit for several years (A.E. Wilson, personal observation).

We did not find any evidence that chemically-assayed secondary metabolites, such as anatoxins and microcystins, had an overall influence on population growth rates of cladocerans and rotifers (Fig. 1.3), but did find some evidence of a toxicity influence on zooplankton survival rates in the secondary analysis of cyanobacteria-versus-starvation experiments (Fig. 1.5). The magnitude of the latter effect, however, was greatly influenced by a single genotype of *M. aeruginosa* (PCC7820), which has been employed repeatedly in the literature and has been shown to produce at least ten different microcystin variants, including microcystin-LR, -LY, -LW, and LF (Robillot et al. 2000). In fact, if we reduce non-independence in the secondary data set by having each genotype be represented by a single observation (by averaging effects sizes within each genotype), the influence of toxicity is no longer statistically significant (non-toxic mean = 0.033, SE = 0.075, $n = 7$; toxic mean = -0.209, SE = 0.080, $n = 21$; $p = 0.109$). As a consequence, we are forced to conclude that, with perhaps the exception of *M. aeruginosa* genotype

PCC7820, there is no strong evidence in the literature to support the generalization that the presence or absence of described cyanotoxins is an important factor driving the poor quality of cyanobacteria as food for zooplankton in general. This does not negate specific cases where zooplankton have been shown to be more negatively affected by diets containing toxic cyanobacteria than non-toxic cyanobacteria (Rohrlack et al. 2001a), but rather suggests that such cases are the exception rather than the rule.

The potential discrepancy in the influence of cyanobacterial toxicity between the population-growth (Fig. 1.3) and survival-only (Fig. 1.5) analyses might be explained by the extraordinary negative effects of cyanobacteria used in the studies included in the secondary analysis (Table 1.2). In these studies, zooplankton fed diets containing toxic cyanobacteria perished prior to reproduction and precluded the ability to calculate population growth rate. Studies incorporated in the primary analyses may have used less toxic strains of cyanobacteria or toxic strains at lower doses, thereby enabling the grazers to reproduce. Thus, the inconsistency between the primary and secondary analyses could be attributable to the effects of a small number of strains of toxic cyanobacteria (e.g., PCC7820) and so may not be a general phenomenon (Fig. 1.5).

Several studies suggest that alternative, undescribed secondary metabolites may be responsible for chemically-mediated cyanobacterial inhibition of grazers (Jungmann 1992; Rohrlack et al. 1999a). Given the results we report (Tables 1.5 and 1.6, Fig. 1.3), perhaps more research effort should be applied to these alternative compounds. In addition, new experimental techniques are needed to better clarify the role of both known and unknown chemical defenses in phytoplankton-zooplankton interactions (see review by Caldwell et al. 2004). As one example, we caution against relying on mouse

bioassays (Carmichael 1992) to classify cyanobacterial taxa or secondary metabolites as "toxic" in the ecological sense of a chemical defense against herbivores, because effects on naïve vertebrates (e.g., mice) may have little relevance with respect to effects on naturally-occurring invertebrates (e.g., zooplankton). Such bioassays are, of course, appropriate for identifying toxins in the context of public health (Carmichael et al. 2001). Lastly, recent studies indicate that grazers can adapt, in an evolutionary sense, to the presence of microcystin-containing cyanobacteria in the diet (Hairston 1999; Sarnelle and Wilson 2005). Thus, it also seems prudent to be more circumspect about the source of the zooplankton strain utilized in laboratory studies of cyanobacteria-zooplankton interactions if the goal is to understand these interactions in nature. We note that few of the studies in our meta-analysis provided detailed information about the habitat from which the zooplankton strains employed were isolated. Given that prolonged exposure to the selective pressures of the laboratory-culture environment may lead to population evolution (Lynch et al. 1991), serious attention should also be paid to the length of time between isolation from nature and use in experiments for both phytoplankton and zooplankton strains.

Cyanobacteria-zooplankton interactions can significantly influence planktonic community dynamics following nutrient and fish manipulations (Sommer 1989). With this in mind, understanding the mechanisms mediating these interactions is critical to effective water quality management. Furthermore, our quantitative survey of the cyanobacteria-zooplankton literature highlights a further need for experiments aimed at teasing apart the effects of cyanobacterial morphology, toxicity, nutrient deficiencies, and interactions among these mechanisms on zooplankton fitness. Finally, copepods are an

important link in aquatic food webs (Sommer 1989), and numerous studies have demonstrated the effects of blue-green algae on copepod filtration rate, egg production, and survival (DeMott et at. 1991). Surprisingly, we found only one paper that presented individual fitness data for a copepod fed a mixture of a cyanobacterium of unknown toxicity and a chlorophyte versus a unialgal diet of the green alga (Twombly et al. 1998). In this study, the cyanobacterial diet was not shown to affect individual grazer fitness. Thus, we encourage future studies aimed at evaluating the effect of cyanobacteria on copepod fitness.

CHAPTER 2

GENETIC VARIATION OF THE BLOOM-FORMING CYANOBACTERIUM, *MICROCYSTIS AERUGINOSA*, WITHIN AND AMONG LAKES: IMPLICATIONS FOR HARMFUL ALGAL BLOOMS.

Abstract

To measure genetic variation within and among populations of the bloom-forming cyanobacterium, *Microcystis aeruginosa*, we surveyed a suite of lakes in the southern peninsula of Michigan that vary in productivity (total phosphorus concentrations ≈ 10 – 100 μg L^{-1}). Survival of *M. aeruginosa* isolates from lakes was relatively low (i.e., mean = 7% and maximum = 30%) and positively related to lake total phosphorus concentration ($p = 0.014$, $r^2 = 0.407$, $n = 14$). In another study, survival rates of *M. aeruginosa* isolates collected from an oligotrophic lake (total phosphorus ≈ 10 μg L^{-1} and DIN:TP ratio = 12.75) differed among five different media types (*G* test $p < 0.001$), with higher survival ($p = 0.003$) in low-nutrient media (28 – 37% survival) relative to high-nutrient media.

Even with the relatively low isolate survivorship that could select against detecting the full range of genetic variation, populations of *M. aeruginosa* were genetically diverse within and among lakes (AMOVA, $\Phi_{pt} = 0.412$, $p = 0.001$), with most clones being distantly related to clones collected from lakes directly attached to the Laurentian Great Lake, Lake Michigan, and culture collection strains collected from Canada, Scotland, and South Africa. Ninety-one percent of the 53 genetically unique *M. aeruginosa* clones contained the microcystin toxin gene (*mcyA*). Genotypes with the

toxin gene were found in all lakes, while four lakes harbored both genotypes possessing and genotypes lacking the toxin gene.

Introduction

The effects of grazers or nutrients on harmful phytoplankton blooms (HABs) or HAB toxins show high temporal and spatial variability (Paerl 1988; Park et al. 1998; Chorus 2001; Raikow et al. 2004; Sarnelle et al. 2005). One source of this variation could be genetic dissimilarity among HAB populations. For example, toxic and nontoxic genotypes within a HAB species might dominate in different habitats and at different times, which could lead to variation in, for example, the ability of consumers to control HABs. However, few studies have measured the genetic composition of HAB populations across time (Laamanen et al. 2002; Kurmayer and Kutzenberger 2003) or space (Bittencourt-Oliveira et al. 2001; Janse et al. 2004), limiting our ability to assess the degree to which environmental variation may select for genotypes with different ecological traits.

In freshwater systems, HABs are largely caused by cyanobacteria in the genera: *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Microcystis*, and *Oscillatoria.* Among these taxa, *M. aeruginosa* is one of the most ecologically damaging species due to its prevalence in water bodies that vary in nutrient loading and its degree of toxicity to aquatic and terrestrial organisms (Carmichael 1992; Chorus and Bartram 1999). Further, we note recent reports showing that the ongoing invasion of freshwaters in North America by the filter-feeding zebra mussel, *Dreissena polymorpha*, is causing an increase in *M. aeruginosa* in low-nutrient lakes (Vasconcelos 1994; Smith et al. 1998; Sarnelle et al. 2005). Due to the present, and potentially increasing, importance of *M. aeruginosa*, we focused our efforts on the genetic diversity of this species.

Cyanobacteria may use a suite of strategies, including morphology and intracellular toxins, to reduce herbivory by filter feeders (Porter and Orcutt 1980; de Bernardi et al. 1981; Lampert 1987). Of these traits, toxic secondary metabolites are the most frequently studied, and recent analytical techniques have identified genes responsible for the production of microcystins, a group of toxins produced by *M. aeruginosa* (e.g., *mcyA*, *mcyB*) (Tillett and Neilan 2000). Microcystins are cyclic peptides that have been shown to be potent hepatotoxins for rodents and humans (Carmichael et al. 2001) and are considered by many to be grazing deterrent compounds (Lampert 1981a,b). If these compounds are grazing deterrents, it might be expected that intense selective herbivory by freshwater grazers, like cladocerans and invasive mussels, would favor genotypes containing microcystin genes. Selection for these toxic genotypes could thus create cascades with major implications on human health (Carmichael 2001).

Traditionally, assessment of diversity within *M. aeruginosa* has focused on morphological variation, such as colony shape and cell size (Vezie et al. 1997). However the concordance between morphological variation and genetic variation is often not clear, especially in microbe populations (Janse et al. 2004). Recent advances in molecular techniques (e.g., *nifH*, *cpcBA*-IGS, nucleotide sequences and HIP-PCR) now allow for extensive examinations of genetic differences among harmful phytoplankton genera, species, and strains within a species (Neilan et al. 1995; Neilan et al. 1997; Koksharova and Wolk 2002; Ouellette and Wilhem 2003). However, many of these studies have focused on strains of cyanobacteria from culture collections (Neilan 1995, Neilan et al. 1997, Otsuka et al. 1999; but see Lyra et al. 2001, Neilan et al. 2003), leaving few reports of the genetic diversity in natural HAB populations (Kurmayer and Kutzenberger 2003;

Janse et al. 2004). Of the latter studies, only a handful provide details about isolate survivorship after sample collection and initial culturing (Hayes and Barker 1997; Postius and Ernst 1999; Rohrlack et al. 2001b; but see Gallagher 1980; Shankle et al. 2004). Many field-collected genotypes may do poorly in culture because abiotic conditions in the lab may not closely match environmental conditions in nature. Limited isolate survivorship may severely constrain assessments of genetic diversity because only a few isolates may be identified, and these may be closely related. It is reasonable to hypothesize that the number of culturable genotypes from an ecosystem might correlate with how well culture conditions match environmental conditions in that particular ecosystem. However, we are unaware of any past studies describing the effect that such conditions have on algal isolate survivorship. If, for example, isolate survivorship in the laboratory is a function of the match between nutrient concentrations in nature versus in the culture medium, the choice of culture medium should presumably be adjusted for differences in nutrient levels among habitats from which isolates are collected. This would reduce biases introduced by the process of genotype isolation, and so improve cross-system comparisons of genetic diversity; it could also, however, confound comparative laboratory studies of ecological traits if all strains can not be maintained on the same culture medium.

Genetic diversity traditionally has been defined as the percentage of distinct genotypes collected from a sampled population. Although past studies describing genetic variation of phytoplankton have shown both little variability (e.g., 0 – 10%; Brand 1988; Barker et al. 2000; Laamanen et al. 2002) and much variability (e.g., 50 – 100%; Gallagher 1998; Medlin et al. 2000; Bittencourt-Oliveira et al. 2001), recent studies of

cyanobacteria suggest considerable genetic diversity both among sites (Bolch et al. 1996; Kondo et al. 1998; Bolch et al. 1999; Hayes et al. 2002; Neilan et al. 2003; Janse et al. 2004) and within lakes (Watanabe 1996; Postius and Ernst 1999; Bittencourt-Oliveira et al. 2001; Laamanen et al. 2002; Janse et al. 2004). In contrast, three recent studies suggest low genetic diversity for certain cyanobacteria (Hayes and Barker 1997; Barker et al. 2000; Humbert and Le Berre 2001). Such conflicting results could be a function of natural history, recent anthropomorphic habitat alterations, different sampling and genetic analysis techniques, or culture conditions that bias results in favor of only a few culturable genotypes. We suggest that more thorough investigations of the genetic makeup of HAB populations among, and especially within, water bodies are necessary to better predict and understand the genesis of HAB events. To our knowledge, this study presents the most extensive dataset describing the genetic composition of *M. aeruginosa* isolates within and among freshwater habitats.

In this paper, we address the following questions: (1) Is there potential isolate bias with respect to the match between nutrient concentrations in standard algal growth media and environmental nutrient concentrations? (2) Are *M. aeruginosa* populations comprised of one or many genotypes? and (3) If significant within-population genetic variation exists for *M. aeruginosa*, do sympatric genotypes vary in the presence of the microcystin gene (*mcyA*)?

Methods

Study lakes

Fourteen lakes in the lower peninsula of Michigan were sampled for *M. aeruginosa* (Table 2.1). One lake (Gull Lake) was sampled three times from June to August 2000, and all fourteen lakes were sampled in August 2002. The lakes were distributed across a broad range of productivity (≈ 10-100 μg L^{-1} total phosphorus (TP)). Two of the lakes, Bear and Spring Lakes, are directly connected to Lake Michigan. Up to three lakes were sampled per day, and at each lake integrated whole water samples of the mixed layer were collected with a tube sampler for chlorophyll *a* analysis. After collecting particles on Gelman A/E filters, chlorophyll *a* was extracted in 90% ethanol (Nusch 1980) and measured via fluorometry with acid correction. Nutrient data and morphometric estimates were obtained from previous studies (Raikow et al. 2004; Wetzel and Likens 1991; L. Knoll, C. Scheele, and S. Hamilton personal communication).

Collection, isolation, and culturing of *Microcystis aeruginosa*

M. aeruginosa colonies were collected with a zooplankton net (30 cm diameter and 100 μm mesh) towed horizontally near the surface. The contents of all tows from each lake were poured into a plastic container and stored in a cooler with lake water until returning to the lab. In 2000, colonies were isolated on the same day as collection. In 2002, multiple lakes were sampled on a single day, so colony isolation was completed the following day. For overnight storage, samples were poured into individual 1 L glass containers and provided with gentle aeration. Floating colonies with features

Table 2.1. Limnological characteristics of the study lakes.

Lake	County (Michigan)	Total phosphorus ($\mu g\ L^{-1}$)	Chlorophyll ($\mu g\ L^{-1}$)[a]	Surface area (hectares)[b]	Maximum depth (meters)	Mean depth (meters)
Bear	Muskegon	66.3	38.6	168[c]	3.7[c]	2.1[b]
Clark	Jackson	17.8	2.4	227	16.8[c]	3.6
Diamond	Cass	23.7	5.3	420	19.5[c]	5.1
Gilkey	Barry	17.1	4.2	32	9.4	5.0
Gravel	Van Buren	22.4	3.8	123	9.5[c]	5.6
Gull	Kalamazoo	19.7	2.0	794	33.5[c]	12.4
Hudson	Lenawee	48.8	55.2	181	9.1[c]	3.1
Magician	Cass	24.6	4.0	202[c]	17.4[c]	2.1[b]
Pine	Barry	27.2	5.5	275	10.4[c]	3.2
Portage	Kalamazoo	28.3	3.6	73[c]	10.4[c]	4.5[e]
Round	Van Buren	35.3	2.9	76	8.2[c]	2.3
Spring	Ottawa	101.8	25.0	424[c]	14.3[c]	6.2[e]
Swan	Allegan	87.0	80.2	81[c]	8.5[c]	3.7[e]
Warner	Barry	12.8	3.8	128	14.0[d]	6.8[d]

[a] = data from 1998-1999 lake survey (Raikow et al. 2004)
[b] = unpublished data from 2000 lake survey
[c] = from STORET database
[d] = personal communication; Alan Tessier, Michigan State University
[e] = calculated from following formula (volume/surface area at zero depth) (Wetzel and Likens 1991)

characteristic of the *M. aeruginosa* morphotype (Watanabe 1996) were pipetted through several wells of either distilled water (isolates in 2000) or BG-11 algal medium (isolates in 2002; media recipe of Vanderploeg et al. 2001) on a well plate, and finally placed into test tubes filled with 15 mL of BG-11 medium.

The examination of culture bias was confined to 2002, where between 40 and 80 colonies were isolated per lake (Table 2.2). All test tubes were placed into an incubator maintained at 23-24°C with a 16:8 hour light:dark cycle, which closely matched the environmental conditions of the study lakes at the time of collection. Culturable isolates were transferred to 125 mL flasks filled with sterile media approximately every four to eight weeks. Isolates that survived the culture process were recorded at each transfer. Although strains were capable of perishing at any time, we conducted our final isolate survival check on 5 February 2003 (Table 2.2 and Fig. 2.1) which allowed any slow-growing isolates ample time (≈ six months) to thrive. Although most strains were unialgal, a few strains were maintained with associated rod-shaped bacteria (e.g., Bear W, Bear AY, Bear AG, Clark DV, Spring CW) or green algae (e.g., Bear AI, Portage EJ, Round S, Round V), thus they were clearly not axenic. Minor contamination of this type was not a problem in this study because cyanobacteria-specific genetic analysis methods were used (HIP-PCR methods below). Four strains of *M. aeruginosa* purchased from two culture collections (University of Texas Culture Collections strains 2385, 2664, and 2667; Pasteur Culture Collection strain 7820) were also genetically analyzed to provide a frame of reference for the field-collected isolates.

Test of nutrient media on isolate survival

To examine the effect of algal nutrient medium on *M. aeruginosa* culturability, we isolated 32 separate colonies from an oligotrophic lake (Gull Lake, Kalamazoo County, Michigan) into each of five media types (pH ≈ 7.5); (1) autoclaved BG-11 (2000 μM N as NH_4^+ and 180 μM P), (2) filter-sterilized BG-11 (2000 μM N as NH_4^+ and 180 μM P), (3) autoclaved BG-11 with reduced N (500 μM N as NH_4^+ and 180 μM P), (4) autoclaved WC-S with NH_4 as the nitrogen source (500 μM N and 19 μM P), and (5) autoclaved WC-S with NO_3 as the nitrogen source (500 μM N and 19 μM P). These media types were chosen in part because we suspected that high NH_4^+ concentrations might be inhibitory to some genotypes. *M. aeruginosa* was collected on 27 September 2002, and colonies were isolated over the following two days using the techniques described previously. We isolated half of the colonies (16 colonies x 5 media types) on the day of collection, and half the following day. Survivorship was determined by visually inspecting tubes for growth on 8 October 2002 (days 10-11) and again on 6 November 2002 (days 40-41).

Genetic analyses

Prior to sample collection for genetic analyses, cultures were transferred to acid-washed flasks and allowed to grow until a sufficient population was established. Cultures were then lyophilized in individual 2 mL centrifuge tubes and stored dry until analyzed.

DNA extraction

DNA extraction was performed using a modification of the bacterial lysis method of Tillet and Neilan (2000). The lyophilised *Microcystis* sample was resuspended in 500

µl XS buffer containing 0.01% (w/v) potassium ethyl xanthogenate (Fluka Chemika), 800 mM ammonium acetate, 100 mM Tris/HCl pH 8.0, 20 mM EDTA and 1% (w/v) SDS. The suspension was incubated at 70°C for 30 min with occasional vortexing and cooled on ice for 30 min. The tube was centrifuged at 12 000 × *g* at 4°C for 10 min and then the supernatant, containing genomic DNA, was carefully removed into a new microcentrifuge tube. The DNA was precipitated by the addition of an equal volume of 2-propanol and incubated at room temperature for 10 min. The DNA was collected by centrifugation at 12 000 × *g* for 20 min at 4°C. The resulting pellet was washed with 70% (v/v) ethanol, dried using a vacuum desiccator and then resuspended in 200 µl TE buffer (10 mM Tris/HCl pH 8.0, 0.1 mM EDTA).

Detection and amplification of microcystin gene (mcyA).

The *mcyA*-specific primer pair QMETF and QMETR was applied to each DNA sample in the following reaction. PCR was carried out in 0.2 ml tubes with a final volume of 20 µl and contained 1 × *F1 Taq* buffer, 0.2 U *F1 Taq* (Fischer Biotech), 200 µM each dNTP, 2.5 mM $MgCl_2$, 5 pmol of each oligonucleotide primer and approximately 50 ng genomic DNA. The amplification profile consisted of an initial denaturation at 95°C for 2 min followed by 30 cycles of 94°C for 10 s, 45°C for 20 s and 72°C for 30 s. This was followed by a final elongation step of 72°C for 2 minutes and final incubation at 20°C.

Amplification of cyanobacterial-specific HIP profile

Samples of DNA were genetically discriminated via highly iterated palindromic polymerase chain reaction (HIP-PCR). The HIP-PCR method has been demonstrated

experimentally to be specific for cyanobacteria (HIP1) (Neilan et al. 2003), except where sequences analogous to the HIP1 octamer were identified in certain halophilic archaebacteria (HIP2) and a thermophile (HIP3) (Robinson et al. 1995). These organisms were unlikely contaminants of our samples since our study sites are temperate, freshwater lakes. For each sample, a PCR was performed with each of the HIP-targeted primers HIP-CA (GCG ATC GCG CA) and HIP-CT (GCG ATC GCG CT). Each reaction was carried out in 0.2 ml tubes with a final volume of 20 µl and contained 1× *F1 Taq* buffer, 0.4 U *F1 Taq* (Fischer Biotech), 200 µM each dNTP, 2.5 mM $MgCl_2$, 10 pmol of the HIPC primer and 1 µl of DNA extract. Thermal cycling consisted of a pre-incubation of 2 min at 94°C, followed by 30 cycles of 10 s at 94°C, 20 s at 40°C and 2 min at 72°C with the ramp-speed between the latter two steps set at 0.1°C min^{-1}. A final incubation at 72°C for 2 min ended the amplification process.

Phylogenetic reconstruction from HIP profiles

The banding patterns produced by the respective HIP-CA and HIP-CT PCRs were scored for the presence or absence of bands in 20 discrete positions for all samples. For each sample, the HIP-CA and HIP-CT scores were concatenated in that order and arranged in a tabular form consistent with that prescribed in the PHYLIP package (Felsenstein 1993). These formatted data were submitted to the program BANDAID (Salmon and Neilan 2004), which calculated pair-wise genetic distances in a format compatible with that prescribed as above. This data were submitted to the NEIGHBOR program of the PHYLIP package which constructed the phylogenetic tree.

Statistical analyses

Linear regression tested for a significant relationship between lake total phosphorus concentration (log TP) and isolate survivorship (log (% + 0.01) or chlorophyll concentration (log chl). An R x C test of independence using *G* test (Sokal and Rohlf 1995) was used to test for survivorship differences among all media types. ANOVA was used to compare isolate survival in the two major different media types (WC media: N = 2 and BG-11 media: N = 3). Analysis of molecular variance (AMOVA, Excoffier et al. 1992) was used to calculate the genetic variation of *M. aeruginosa* within and among populations, and provide pair-wise comparisons of genetic structure between different populations of *M. aeruginosa*. All statistical analyses were performed with Systat 9.01 (SPSS 1998) or GenAlEx 5.0 (Peakall and Smouse 2001). Rejection criterion was set at $\alpha < 0.05$.

Results

Thirteen of the fourteen lakes sampled had culturable isolates. Isolate survival for *M. aeruginosa* collected from the study lakes in August 2002 varied from 0 - 30% (mean ± 1 std. error = 7.4 ± 2.1 %) across all lakes and was positively related to lake total phosphorus concentrations ($p = 0.014$, $r^2 = 0.407$, $n = 14$; Table 2.2 and Fig. 2.1).

We found a significant effect of growth medium on the culturability of *M. aeruginosa* isolates from Gull Lake (*G* test statistic = 38.7, df = 4, $p < 0.001$) with significantly higher survivorship in WC media versus BG-11 media ($p = 0.003$). The highest isolate survival rates were observed in the relatively low phosphorus WC media treatments (37.5% survival WC-S with NH_4; 28.1% survival WC with NO_3) with little survival (0 – 3.7%) in the BG-11 media types that contained higher phosphorus concentrations.

Seventy-nine percent of the 67 cyanobacterial isolates genetically analyzed with HIP-PCR were shown to be genetically distinct. Percent distinct genotypes $\left(\frac{\#\text{ of distinct genotypes}}{\#\text{ of isolates analyzed}} * 100\right)$ from lakes with two or more analyzed isolates ranged from 42% - 100% (mean ± std. error = 78 ± 7.8%). Nine of the ten lakes where two or more isolates were analyzed via HIP-PCR showed at least two distinct genotypes. In addition, in two lakes, Clark and Round, 9 of 9 isolates were genetically distinct. Interestingly, one genotype (Table 2.2 ClarkB02/PineCD02/PineCF02/PineCG02) was observed in two lakes, Clark and Pine, which were separated by 130 kilometers. We did not detect this genotype in any lakes situated near Pine Lake.

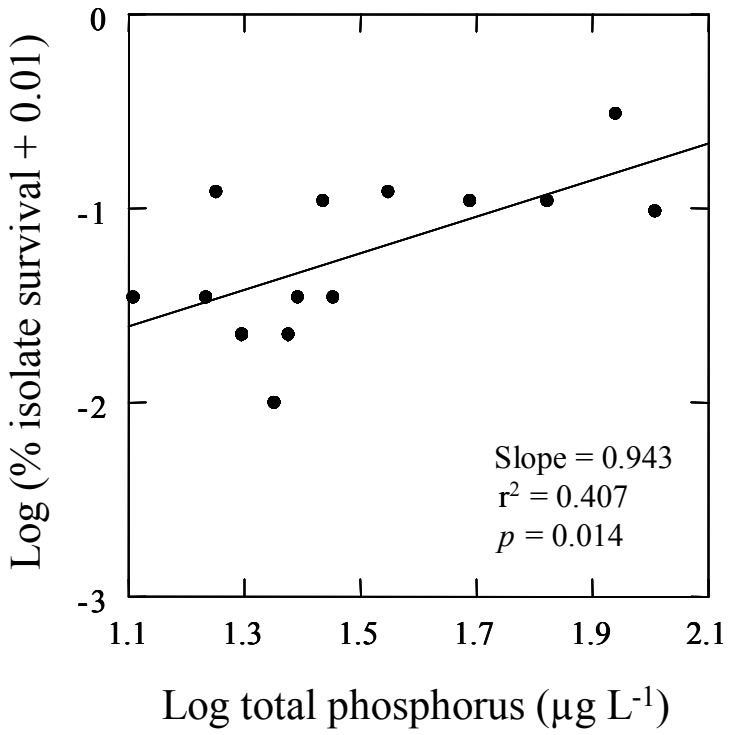

Figure 2.1. Relationship between lake trophic status (measured as total phosphorus concentration (µg L^{-1})) and log (isolate survival + 0.01) (% = # of survivors/# of total isolates collected).

Table 2.2. Summary of results for *M. aeruginosa* isolate survival and genetic analysis. Some isolates perished prior to genetic analysis. N/A = not applicable. N/R = not recorded.

Lake	Sample date	Isolates collected	Surviving isolates	# of clones genetically analyzed	# of distinct genotypes
Bear	08/15/02	80	8 (10%)	7	7 (100%)
Clark	08/17/02	80	9 (11%)	9	9 (100%)
Diamond	08/13/02	80	1 (1%)	0	0
Gilkey	08/05/02	40	1 (3%)	1	1
Gravel	08/09/02	80	0 (0%)	0	0
Gull	06/19/00	N/R	N/A	1	1
	06/29/00	N/R	N/A	8	6 (75%)
	08/23/00	N/R	N/A	1	1
	08/07/02	80	1 (1%)	1	1
Hudson	08/17/02	40	4 (10%)	3	2 (67%)
Magician	08/11/02	80	2 (3%)	2	1 (50%)
Pine	08/05/02	40	4 (10%)	4	2 (50%)
Portage	08/07/02	80	2 (3%)	2	2 (100%)
Round	08/11/02	80	9 (11%)	9	9 (100%)
Spring	08/15/02	80	7 (9%)	6	6 (100%)
Swan	08/09/02	40	12 (30%)	12	5 (42%)
Warner	08/05/02	40	1 (3%)	1	1

Populations of *M. aeruginosa* exhibited significant genetic variation (Table 2.2, Figs 2.2 and 2.3) among lakes (Table 2.3; AMOVA, $\Phi_{pt} = 0.412$, $p = 0.001$), despite the low culturability of isolated colonies (Table 2.2). Interestingly, only 41% of the estimated genetic variance could be explained by *among*-lake variation, while 59% of the genetic variation was explained by *within*-population variation. AMOVA also revealed many statistically significant pair-wise comparisons between *M. aeruginosa* populations in different lakes with each population being genetically distinct from at least three other populations (Table 2.3). The extreme examples were the Gull, Round, and Swan Lake populations, which were genetically distinct from all other cyanobacterial populations assessed ($p \leq 0.05$). The Portage and Hudson Lake populations exhibited the least genetic dissimilarity from other populations (only 3 significant pair-wise comparisons between either of these lakes and the other 9 lakes).

The phylogenetic analysis of the PCR products also suggested much genetic variation within and among lakes (Fig. 3). Isolates from six (Bear, Clark, Hudson, Portage, Spring, and Swan) of the lakes were dispersed on the phylogenetic tree, while cultures from Gull Lake, Pine Lake, and Round Lake tended to be clustered with sympatric clones. Only one lake, Gull Lake, was sampled multiple times, and the same genotype was never collected on more than one date.

Comparing the phylogenetic positions of the *M. aeruginosa* cultures from the study lakes with the four culture collection strains revealed interesting relationships. All culture collection strains grouped near the origin of the tree and were intermixed with several strains from Bear and Spring Lakes, along with one strain from Hudson and Gilkey Lakes. As might be expected, UTEX 2385 and UTEX 2667 (both from Little

Table 2.3. AMOVA output for population genetic comparisons of *M. aeruginosa* clones from the ten study lakes for which multiple clones were analyzed. Φ_{pt} values are shown below diagonal. *p*-values for pairwise comparisons between lakes are shown above diagonal.

Source	df	SS	MS	Estimated variance	% variance explained	Φ_{pt} value	*p*-value
Among Pops.	9	71.07	7.90	1.02	41%	0.412	0.001
Within Pops.	55	80.37	1.46	1.46	59%		

	Bear	Clark	Gull	Hudson	Magician	Pine	Portage	Round	Spring	Swan
Bear	- -	0.013	0.002	0.186	0.050	0.029	0.528	0.001	0.329	0.001
Clark	0.237	- -	0.001	0.097	0.240	0.188	0.248	0.001	0.011	0.002
Gull	0.278	0.327	- -	0.004	0.011	0.003	0.026	0.001	0.005	0.001
Hudson	0.099	0.193	0.467	- -	0.199	0.064	0.405	0.010	0.142	0.006
Magician	0.316	0.074	0.611	0.444	- -	0.060	0.332	0.015	0.100	0.019
Pine	0.212	0.078	0.490	0.391	0.442	- -	0.137	0.002	0.064	0.002
Portage	0.000	0.059	0.424	0.172	0.500	0.000	- -	0.037	0.378	0.024
Round	0.528	0.355	0.612	0.616	0.794	0.648	0.557	- -	0.002	0.001
Spring	0.011	0.296	0.383	0.192	0.363	0.282	0.000	0.475	- -	0.001
Swan	0.392	0.482	0.592	0.563	0.615	0.652	0.550	0.677	0.400	- -

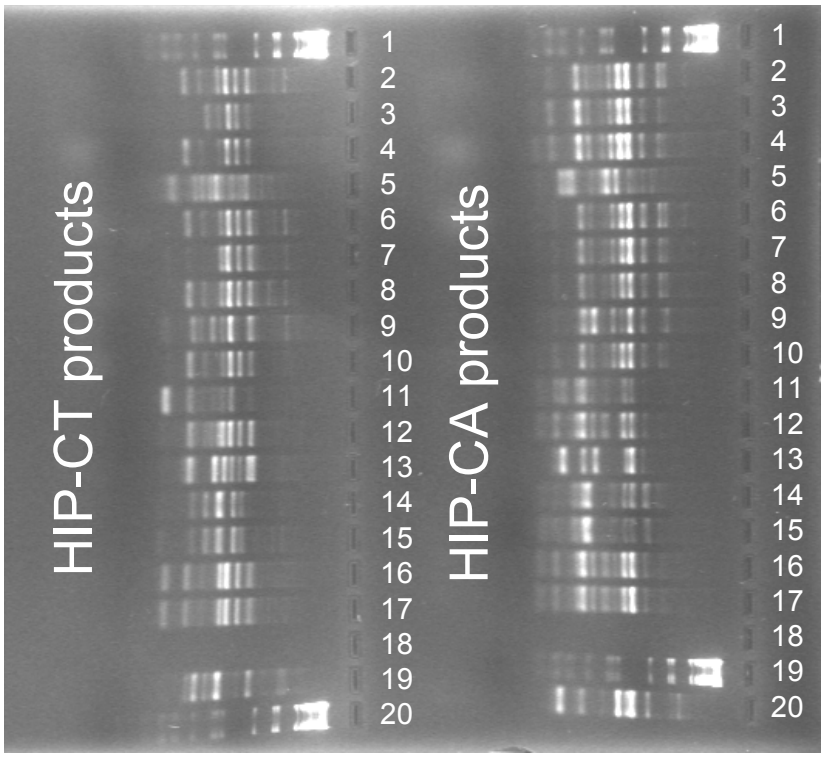

Figure 2.2. Example of PCR gel of HIP products. HIP-CT (left) and HIP-CA (right) samples for *M. aeruginosa* isolates from Clark Lake (lanes 2-10), Gilkey Lake (lane 11), Gull Lake (lane 12), Hudson Lake (lanes 13-15), Magician Lake (lanes 16-17), PCC 7806 (lane 19 HIP-CT, lane 20 HIP-CA), and standards (lanes 1 and 20 HIP-CT; lanes 1 and 19 HIP-CA). Note differences among Clark Lake and Hudson Lake strains.

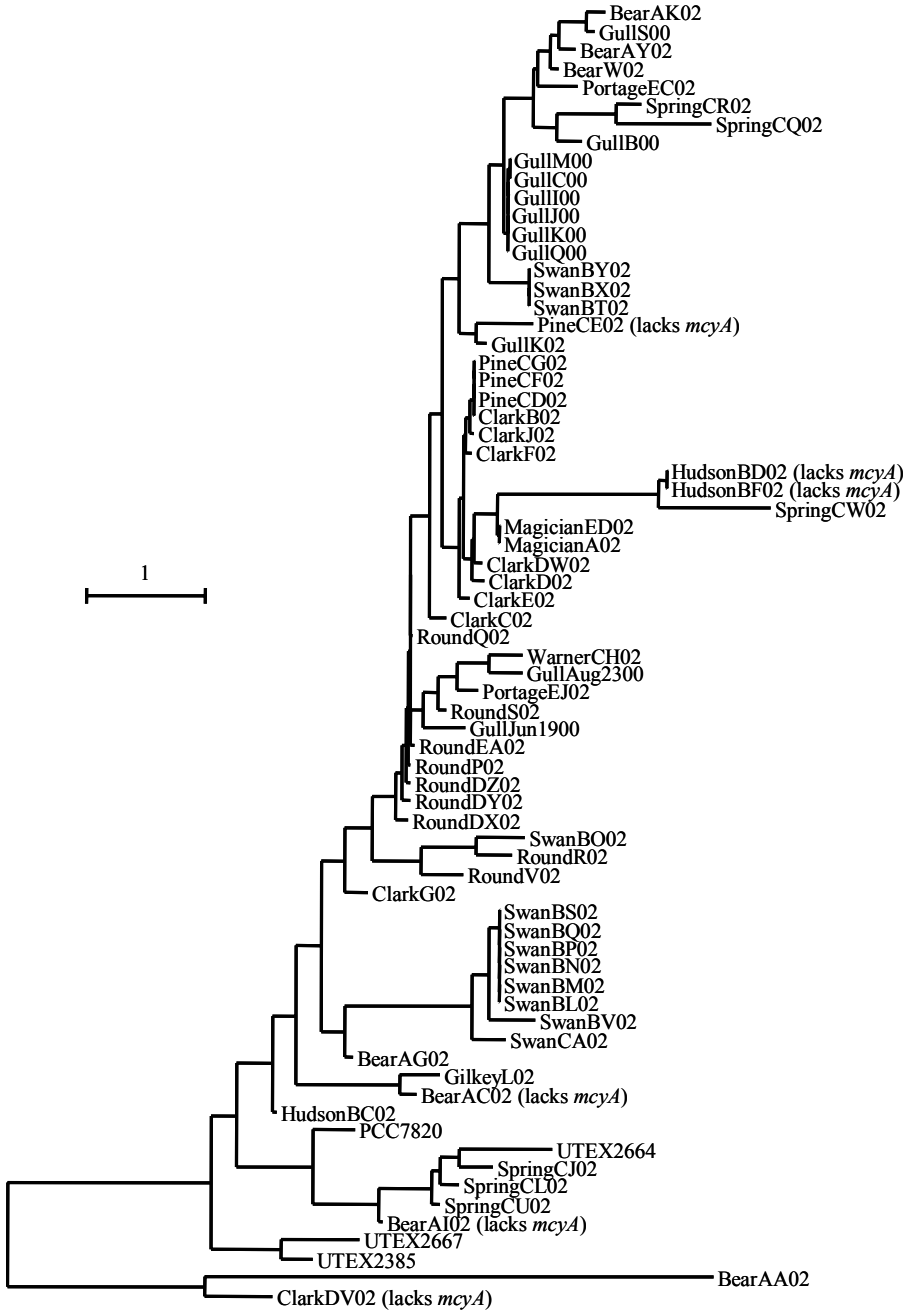

Figure 2.3. Phylogenetic tree for *M. aeruginosa* isolates created by calculating the relative "distances" of PCR products in a pair-wise fashion between the banding patterns of each HIP PCR (BandAid program created by Tim Salmon at UNSW). Toxin gene absence (*mcyA*) denoted by (*mcyA*-). All other strains contain toxin gene. Scale = 1 relative distance unit.

Rideau Lake, Ontario, Canada) were neighbors on the tree. Additionally, UTEX 2664 (South Africa) appears to be closely related to PCC 7820 (Scotland) and several isolates from Bear and Spring lakes. Two isolates, BearAA02 and ClarkDV02, were outgroups, however their colony morphologies suggested that they were *M. aeruginosa*.

The microcystin toxin gene (*mcyA*) was detected in most isolates (92.5%) and unique genotypes (90.6%) and in all 12 lakes that had *M. aeruginosa* genotypes (Table 2.2 and Fig. 2.3). Only five genotypes lacked the toxin gene. Four lakes harbored both toxic and non-toxic genotypes (Bear, Clark, Hudson, and Pine).

Discussion

We found considerable genetic variation within and among populations of the bloom-forming phytoplankter, *M. aeruginosa*, in southern Michigan lakes. Fifty-three of the 67 isolates analyzed via HIP-PCR were shown to be genetically distinct. In the four lakes with larger sample sizes of isolates (six to nine isolates analyzed), virtually all isolates were genetically distinct. We found only one instance of the same genotype being present in two separate lakes (ClarkB02/PineCD02/PineCF02/PineCG02). Although genetic variability of cyanobacteria (Dyble et al. 2002; Neilan et al. 2003), including *M. aeruginosa* (Watanabe 1996; Bittencourt-Oliveira et al. 2001; Janse et al. 2004), has been documented previously, this dataset represents the most comprehensive genetic analysis of *M. aeruginosa* strains collected from within and among lakes in North America (see Janse et al. 2004 for a survey of *Microcystis* morphotypes throughout Europe). Furthermore, we note that if different colony morphologies (e.g., *M. botrys*, *M. flos-aquae*, *M. icthyoblabe*, *M. viridis*, and *M.wesenbergii*) represent different strains within *M. aeruginosa*, then we may have underestimated the genetic diversity of this bloom-forming phytoplankter because we limited our collections to only those morphologies that are typical of *M. aeruginosa* (i. e., our conclusion of "high" diversity is therefore conservative).

Low isolate survival in culture may lead to underestimation of genetic variability, yet few studies have quantified isolate survival. Given the relatively low isolate survival in our study, we caution that genetic variability of *M. aeruginosa* in Michigan lakes may be substantially greater than we report. Newer techniques enable determination of genetic diversity based on freshly-collected individual colonies (Fastner et al. 2001;

Kurmayer and Kutzenberger 2003; Janse et al. 2004), and so eliminate problems related to culture bias. However, genetic analysis of individual colonies precludes culturing (since the colonies are destroyed in the process) to assess isolate survivorship as a function of media type or isolation procedures.

We found that the average survivorship rate observed for isolates collected from 14 lakes (7.4%) was higher than some studies (Postius and Ernst 1999; Rohrlack et al. 2001b) but much lower than others (Gallagher 1980; Shankle et al. 2004). Although our survivorship estimates were low, survivorship of *M. aeruginosa* isolates cultured in a nutrient-rich medium was positively related to ambient nutrient concentrations found in our study lakes. In other words, isolates from oligotrophic lakes showed lower survivorship when cultured in nutrient-rich algal medium than isolates collected from more eutrophic lakes (Fig. 2.1, Table 2.2). Our observation that *M. aeruginosa* isolates from an oligotrophic lake survived better in less-rich WC media versus more-rich BG-11 media provides further support for this pattern. Such results have important implications for scientists studying the population genetic structure of phytoplankters in freshwater, and possibly marine, habitats that vary in nutrient concentrations. Furthermore, scientists interested in collecting isolates from the field to be used in later experiments may be advantaged by choosing an algal medium based on the nutrient regime of the waterbodies sampled.

Our lake survey revealed substantial genetic diversity within *M. aeruginosa* both within and among lakes. Bittencourt-Oliveira et al. (2001) provided similar results for *M. aeruginosa* strains from four Brazilian reservoirs. In that study, nine distinct genotypes were collected from four sites with at most 6 genotypes collected from one site and one

specific genotype collected from two sites. Additionally, one unique genotype of *M. aeruginosa* was collected from the same site over time and along a depth gradient at the same sampling time (Bittencourt-Oliveira et al. 2001). Most recently, Janse et al. (2004) surveyed the genetic diversity of 107 *Microcystis* colonies (seven morphospecies) from 15 European lakes and characterized 59 distinct genetic classes - demonstrating significant genetic variation within and across habitats. For example, all but one *Microcystis* population (93%) was comprised of at least two distinct classes and 24% (14 of 59 classes) of the *Microcystis* classes were found from at least two different lakes with one group being found in lakes located in the Czech Republic, Germany, Italy, and Scotland. Additionally, no relationship was found between morphospecies designation and genotypic class. Although our study was more restricted geographically, we also found substantial genetic variation within and among *Microcystis* populations. Thus, populations of *M. aeruginosa* from diverse habitats and within individual lakes are genetically heterogeneous, which could have major ecological implications for the detection, development, mitigation, and ecological impacts of HABs.

Recent reports have documented higher genetic similarity for nearby bacterial communities versus more distant communities (Green et al. 2004; Horner et al. 2004). Although few studies have determined how genetic variance is partitioned within and among habitats for phytoplankton, AMOVA estimated that 41% of the variation for our *M. aeruginosa* clones was attributed to among-lake differences while the remaining variation (59%) could be explained by within-lake differences. We found no other studies that used this statistical technique to partition variance among and within populations for cyanobacteria, but our results are consistent with those provided by

Shankle et al. (2004) for dinoflagellate populations off the coast of southern California. They found that the genetic variation for *Prorocentrum* populations was almost entirely attributed to within population differences (93%) while very little of the variation could be explained by among population differences (10%). Although the surveyed habitats in these two studies are very different biologically, chemically, and physically, both studies show that most of the genetic variation could be attributed to within–habitat differences with less variation being explained by among-habitat differences.

Four strains of *M. aeruginosa* from three freshwater lakes in North America, Africa, and Europe maintained at two culture collections, were genetically analyzed as reference strains (Fig. 3). The culture collection strains were most related to each other and to several, but not all, strains of *M. aeruginosa* from Bear and Spring Lakes. The similarity between the culture collection strains and those strains positioned near the origin of the phylogenetic tree from Bear and Spring Lakes could be due to the similar nutrient levels in these waterbodies. Bloom-forming cyanobacteria are most prevalent in eutrophic lakes, so it is not surprsing that the culture collection strains used in this study were isolated from mesotrophic to hypereutrophic lakes (Bryant et al. 1997; Forrest et al. 2002).

We also found that populations of *M. aeruginosa* in four lakes contained genotypes with and without one of the genes responsible for microcystin production. Several other studies provided similar results (Vasconcelos 1994; Watanabe 1996, Kurmayer and Kutzenberger 2003; Janse et al. 2004). Vezie et al. (1997) isolated strains of *M. aeruginosa* from three freshwater sites in France in 1994 and showed that at least one strain from each site produced microcystins and at least one strain did not. Kurmayer

and Kutzenberger (2003) showed seasonal variation for the occurrence of a microcystin gene (*mcyB*) in a natural population of *M. aeruginosa* in Lake Wannsee from June 1999 to October 2000 with the lowest proportion of colonies containing the toxin gene occurring in the Spring. Janse et al. (2004) showed that at least 7 of the 15 European lakes surveyed contained sympatric *Microcystis* classes that either tested positive or negative for microcystin or its biosynthetic genes. Finally, Welker et al. (2003) used two methods (agar plating and liquid media) to isolate colonies of *M. aeruginosa* from Lake Müggelsee and showed that both techniques produced contrasting results when evaluating strains for toxin production. Agar plating selected for non-toxic strains (96% non-toxic strains) while liquid media selected for toxic strains (5% non-toxic strains). Thus, different isolation and culturing techniques could select for toxic or non-toxic strains of *M. aeruginosa*, and these differences should be considered when developing an isolation/culturing protocol for HAB species.

In conclusion, we show that *M. aeruginosa* populations in the southern peninsula of Michigan are genetically diverse and that isolate survival in a nutrient-rich culture medium is positively related to total phosphorus concentrations of the source lakes. We encourage future studies aimed at addressing population-level genetic diversity of harmful algal species both in space and time (see Postius and Ernst 1999; Bittencourt-Oliveira et al. 2001; Hayes et al. 2002). Such information could be useful at predicting and mitigating future HABs and explaining unusual phenomona such as seasonal variation in the toxin type and content of lakes (Vasconcelos 1994; Park et al. 1998; Oh et al. 2000; Chorus 2001).

CHAPTER 3

INTRASPECIFIC VARIATION IN THE GROWTH AND MORPHOLOGY OF THE BLOOM-FORMING CYANOBACTERIUM, *MICROCYSTIS AERUGINOSA*: FASTER-GROWING COLONIES GET LARGER

Abstract

This study represents a comprehensive survey of phenotypic and growth rate variation across genotypes of the harmful phytoplankter, *Microcystis aeruginosa*. In the laboratory, we documented large variation in maximum population growth rate, morphology, and production of the toxin, microcystin, for thirty-two conspecific *M. aeruginosa* strains isolated from 12 lakes in Michigan. Unlike many previous studies, most of the *M. aeruginosa* strains we studied exhibited the colonial morphology diagnostic of the species in nature. Maximum population growth varied significantly across strains (range = 0.12 to 0.44 day^{-1}) and populations, but did not differ when comparing single-celled and colonial strains. Growth rates were positively correlated with colony surface area, but not with toxin production. Cell diameter and colony size varied significantly across *M. aeruginosa* strains, and colony size differed significantly across populations. Variation in cyanobacterial morphology and growth rate has been shown to affect consumption by grazers and competitive dominance among multiple species of phytoplankters, but few data address the amount and consequences of phenotypic variation within a species of a bloom-forming cyanobacterium. Such variation could have important ecological implications, such as the promotion and/or

control of harmful algal blooms by influencing interactions with other phytoplankters and consumers.

Introduction

Microcystis aeruginosa is a ubiquitous, bloom-forming cyanobacterium that causes considerable ecological, economic, and aesthetic damage to freshwater and estuarine ecosystems via the formation of surface scums, production of intracellular toxins (e.g., microcystins), and creation of anoxic conditions when blooms degrade (Carmichael 1994; Christoffersen 1996; Watanabe et al. 1996; Chorus and Bartram 1999). In extreme cases, human sickness and death have been related to blooms of cyanobacteria, including *Microcystis* (Carmichael et al. 2001). Although *Microcystis* frequently dominates nutrient-rich habitats during warm summer months (Reynolds et al. 1981; Watanabe et al. 1996; Zurawell et al. 2005), recent observational and experimental studies in low-nutrient lakes show that even oligotrophic lakes are prone to blooms of *M. aeruginosa* as a result of invasion by exotic zebra mussels (*Dreissena polymorpha*) (Raikow et al. 2004; Sarnelle et al. 2005; Knoll et al. in press).

Microcystis and herbivorous grazers interact in complex ways (Lampert 1987; Lürling 2003a; Sarnelle et al. 2005) that may be attributed to genetic and/or physiological variation within and among these prey and consumer populations (Hairston et al. 1999; Sarnelle and Wilson 2005; Wilson and Hay in prep). Cyanobacteria may use one or a suite of different defense mechanisms to deter herbivores, including intracellular toxins, large growth forms such as filaments and colonies, and buoyancy regulation (Reynolds 1984; Watanabe et al. 1996). With respect to toxicity, populations of *Microcystis* contain strains that possess or lack the genes for producing microcystins (Vézie et al. 1997; Kurmayer et al. 2003; Janse et al. 2004; Wilson et al. 2005), which are a class of secondary metabolites that inhibit protein phosphatase inhibitors and promote tumors in

mammals (Carmichael 1992). Microcystins are produced by several cyanobacterial genera, such as *Anabaena*, *Microcystis*, *Nostoc*, and *Planktothrix* (Carmichael 1992). Although these compounds are widely considered harmful to grazers, a recent meta-analysis of the freshwater zooplankton-cyanobacteria interaction literature suggests otherwise (Wilson et al. in press).

As an alternative defense mechanism, cyanobacteria may lessen their susceptibility to grazing by growing as large colonies (Webster and Peters 1978). Colonial forms of cyanobacteria can be difficult for some zooplankton to handle or can interfere with zooplankton filtering appendages (Webster and Peters 1978; Gliwicz 1990). More importantly, the presence of cyanobacterial filaments can influence community dynamics of zooplankton by favoring smaller grazers over larger grazers (Fulton and Paerl 1988; Gilbert 1990). Mechanisms driving these changes are not fully understood, but it is possible that smaller grazers avoid large cyanobacteria while preying on associated bacteria or other phytoplankters found within the cyanobacterial matrix (Paerl et al. 2001). Such changes in community composition via physiological and genetic differences among strains of cyanobacteria likely affect trophic interactions and have important implications on community and ecosystem dynamics; however this possibility has yet to be experimentally addressed in the field.

Recent studies have highlighted significant genetic variation within cyanobacteria (Hayes and Barker 1997; Vézie et al. 1997; Bolch et al. 1999; Dyble et al. 2002), the genus *Microcystis* (Bittencourt-Oliveira et al. 2001; Kurmayer and Kutzenberger 2003; Janse et al. 2004), and the species *M. aeruginosa* (Wilson et al. 2005), in Europe, South America, and North America. However, limited information exists regarding ecological

or physiological variation among conspecific strains of *Microcystis* or cyanobacteria in general (Reynolds et al. 1981; Robarts and Zohary 1987; Saker and Neilan 2001). Most studies examining the effects of environmental manipulations on physiological traits of cyanobacteria focus on one or a few congeneric or conspecific strains (Saker and Neilan 2001; Lyck and Christoffersen 2003; Wiedner et al. 2003; Lyck 2004). Results from these studies show that individual cyanobacterial strains exhibit vast variation in physiological responses to temperature, light, salinity, and macronutrient manipulations (Lee and Rhee 1999; Oh et al. 2000; Saker and Neilan 2001; Hobson and Fallowfield 2003), however some of these studies used single-celled strains of cyanobacteria that lack phenotypic characteristics commonly observed in nature (e.g., colonial morphology for *Microcystis*). Extrapolating results from studies using these types of atypical strains to naturally occurring clones could be problematic given that cyanobacterial morphology affects nutrient uptake rates, sinking rate, buoyancy, and susceptibility to grazing (Reynolds et al. 1981; Lampert 1987; Wilson et al. in press).

One of the most important ecological characteristics of a species is its maximum population growth rate (Sibly and Hone 2002) which integrates across numerous physiological responses, provides direct information about population dynamics, and influences how specific genotypes interact with other organisms in their community. However, maximum population growth rates are observed in resource-saturated, competitor-less, and predator-less environments, so caution should be used when trying to extrapolate growth rate estimates measured in the laboratory to those observed in the field. Interestingly, relatively few studies have compared maximum population growth rate across multiple strains of *Microcystis* and even fewer studies have estimated

maximum growth rates using colonial *Microcystis* (see review by Robarts and Zohary 1987). Since the environment can select for different traits, we hypothesized that the growth rate of individual *M. aeruginosa* genotypes would be (1) positively correlated with the productivity of the lake from which the clones were collected since lake nutrient levels could select for specific cyanobacterial strains that perform best at *in situ* nutrient concentrations found in the lake of strain collection, but (2) negatively correlated with colony size since large forms of cyanobacteria should experience more self-shading than smaller colonies and since nutrient transport outside colonies to intra-colonial parts should be more efficient for smaller colonies. To address these hypotheses, we measured maximum population growth rate and morphological characteristics of 32 genetically distinct strains of *M. aeruginosa* and used these data to determine: (i) the magnitude of phenotypic variation in maximum growth rate, cell size, colony surface area, and toxin content among sympatric and allopatric strains of *M. aeruginosa*, (ii) the relationship between strain growth rate and phenotypic traits of the strains (cell size, colony surface area, and toxin content), (iii) the relationship between growth rate and the productivity (measured as chlorophyll and phosphorus concentrations) of the lakes from which the strains were collected, and (iv) the relationship between strain growth rate and genetic relatedness.

Methods

Experimental design

Maximum population growth rates were estimated for thirty-two genetically distinct strains of *Microcystis aeruginosa* isolated from 12 lakes in the southern peninsula of Michigan (see Wilson et al. [2005] for details about isolate collections, lakes, and genetic analysis). Most of the strains continued to maintain their characteristic irregular colony growth habit in culture; however some of the strains were single-celled during the growth experiments, possibly due to frequent mixing. Light (250 μmol of photons m^{-2} s^{-1} radiating from cool white fluorescent lights at 18:6 light:dark cycle) and nutrient conditions (2000 μM $NaNO_3$, 180 μM K_2HPO_4; BG-11 medium, [Vanderploeg et al. 2001]) were at saturating levels and the experiment was conducted in a temperature controlled room at 25 °C.

All *M. aeruginosa* clones had been routinely cultured at 22 °C in BG-11 medium with minimal light exposure to reduce growth, but were allowed to acclimate to the experimental conditions for 5 weeks prior to the start of the experiment. Before initiating the experiment, all stock culture concentrations (μm^3 biovolume ml^{-1}) were determined with a Coulter Multisizer III (100 μm aperture tube) after homogenizing each flask by vigorous hand mixing. Either 4 (n = 30 strains), 8 (n = 1 strain) or 12 (n = 1 strain) replicate sterilized 250 ml flasks were filled with 150 ml of sterile BG-11 medium and inoculated with approximately 1 x 10^3 μm^3 biovolume ml^{-1} of each *M. aeruginosa* clone. Mean particle biovolumes did not differ (p = 0.755) across all 32 clones on day 0 as determined using a Coulter Multisizer III. Replicate numbers differed across strains because previous experiments suggested that the physiological traits for two strains, i.e.,

RoundDX02 and SpringCJ02, were highly variable, so we increased sample sizes to enhance confidence in our measurements for these strains. Flasks were stirred by hand then rotated among positions in the environmental chamber twice daily to homogenize light intensities among the flasks. Five ml samples were collected on days 0, 2, 5, 7, and 9 and preserved in 1% Lugol's iodine solution for later cell, colony, and total biovolume measurements. *M. aeruginosa* in 12 of the 140 total flasks used in this study did not grow, so these flasks were excluded from the analyses, leaving 3 – 10 replicate flasks per strain.

Growth rate, cell size, colony size, and microcystis measurements

Subsamples (1 ml) were collected from each sample, mixed with a Vortex mixer, and added to 24 ml of diluent before duplicate biovolume estimates were determined using a Coulter Multisizer III. Duplicates were averaged and maximum population growth rates were determined by fitting log transformed biovolume data against sample dates to calculate a least squares regression for each *M. aeruginosa* clone. A few (6% of total samples) outlier data points were identified (studentized residuals ≥ 3) and removed before calculating growth rates. Cell diameters and colony surface areas were measured with a compound microscope (1000 x and 400 x, respectively) for all preserved samples collected on day 9 at the end of the experiment. Microcystin concentration (ng toxin (μg dry biomass)$^{-1}$) for each *M. aeruginosa* strain was determined via an enzyme-linked immunosorbent assay (ELISA) (An and Carmichael 1994). Cells were collected on glass-fiber filters, dried and weighed to calculate dry biomass, stored frozen, and extracted in 75% aqueous methanol prior to ELISA analysis. One strain (BearAC02) initially considered to be non-toxic via genetic analysis (Wilson et al. 2005) contained

quantifiable levels of microcystins (0.117 ng (μg dry biomass)$^{-1}$) suggesting an error with the detection of the toxin gene or toxin analysis for this strain. No other inconsistencies between toxin gene presence/absence and toxin content were found for the remaining 31 *M. aeruginosa* strains.

Phylogenetic reconstruction from HIP profiles

All *M. aeruginosa* strains used in this study are genetically distinct as determined by highly iterated palindromic PCR (HIP-PCR; see Wilson et al. [2005] for more details). Briefly, data produced by the HIP-PCR analysis were scored for the presence or absence of bands in 20 discrete positions and analyzed with BANDAID (Salmon and Neilan 2004) to calculate pairwise genetic distances between the strains. These data were submitted to the nearest neighbor program of PHYLIP (Felsenstein 1993) which produced the dataset used to construct the phylogenetic tree using TREEVIEW (Page 2001).

Statistical analysis

Analysis of variance (ANOVA) evaluated morphological and physiological differences among the *M. aeruginosa* strains and populations. Replicate flasks were considered independent when testing hypotheses across genotypes, while the mean response for each strain across replicate flasks was considered an independent observation when contrasting responses across populations. Pearson's product moment correlation determined how well growth rates related to physiological features of the strains (i.e., cell size, colony area, toxin content) and to the lakes' limnological characteristics (i.e., phosphorus concentration, chlorophyll concentration, depth, and surface area). Data were log transformed to reduce heteroscedasticity and standardize

variance, when needed. All analyses were performed with SYSTAT 9.01 (SPSS 1998). Rejection criterion was set at $\alpha < 0.05$.

Results

Maximum population growth rates were linear over the 9 day laboratory experiment and differed significantly among the 32 *M. aeruginosa* strains (Fig. 3.1, $p < 0.001$). When analyzed by lake of origin, mean growth rates also differed significantly among lakes (Table 3.1, $p = 0.034$). Growth rates ranged from 0.12 day^{-1} to 0.44 day^{-1} (Fig. 3.1) with an average growth rate across all strains of 0.23 day^{-1}. Growth rates were similar for single-celled (0.23 ± 0.02 day^{-1} mean ± standard error, $n = 13$) and colonial strains (0.22 ± 0.02 day^{-1} mean ± standard error, $p = 0.710$, $n = 19$), suggesting that colony formation was not costly in terms of growth rate for *M. aeruginosa* strains, at least over 9 days. Cell diameters ($p < 0.001$) and colony surface areas ($p = 0.005$ after removing one very large outlier [studentized residual = -3.5]; including outlier $p = 0.070$) also differed across the distinct *M. aeruginosa* strains. Colony surface areas differed ($p = 0.013$) among lakes (Table 3.1), while cell diameters did not ($p = 0.574$). Across all strains, cell diameters averaged 4.72 μm with a range of 3.11 μm to 5.20 μm and colony surface areas averaged 7672 μm^2 with a range of 1524 μm^2 to 24133 μm^2. Microcystin concentrations across all non-toxic and toxic strains ranged from 0.00 ng (μg dry mass)$^{-1}$ to 0.98 ng (μg dry mass)$^{-1}$, and averaged 0.24 ng (μg dry mass)$^{-1}$ (Fig. 3.2). Strains lacking the toxin gene, *mcyA*, (HudsonBD02 and PineCE02), did not grow consistently faster or slower than those strains possessing the toxin gene (Figs. 3.1 and 3.2), although there was a nearly significant ($p = 0.054$) variance in microcystin concentrations for toxic *M. aeruginosa* strains from different populations (Table 3.1). Additionally, there was no correlation of toxin content with growth rate for toxic strains (Table 3.2, Fig. 3.2;

Pearson's $r = 0.059$, $p = 0.761$, $n = 29$), suggesting no trade-off of growth rate with increased toxin production exists for *M. aeruginosa* strains.

Correlations between physiological properties of the *M. aeruginosa* strains and environmental characteristics of the study lakes from which the strains were collected provided few significant relationships (Table 3.2, Fig. 3.1). Besides the relationship between maximum population growth rate and colony surface area, significant positive relationships were found for colony surface area and lake total phosphorus (Table 3.2, Pearson's $r = 0.460$, $p = 0.047$) and chlorophyll concentrations (Table 3.2, Pearson's $r = 0.691$, $p = 0.001$).

Sympatric *M. aeruginosa* strains tended to be more closely related than did strains from separate lakes (Fig. 3.3). Growth rates for related strains also tended to be similar, but exceptions did occur – i.e., the fastest growing strain BearAC02 (growth rate = 0.44 day^{-1}) clustered genetically with the slow growing strain GilkeyL02 (growth rate = 0.19 day^{-1}).

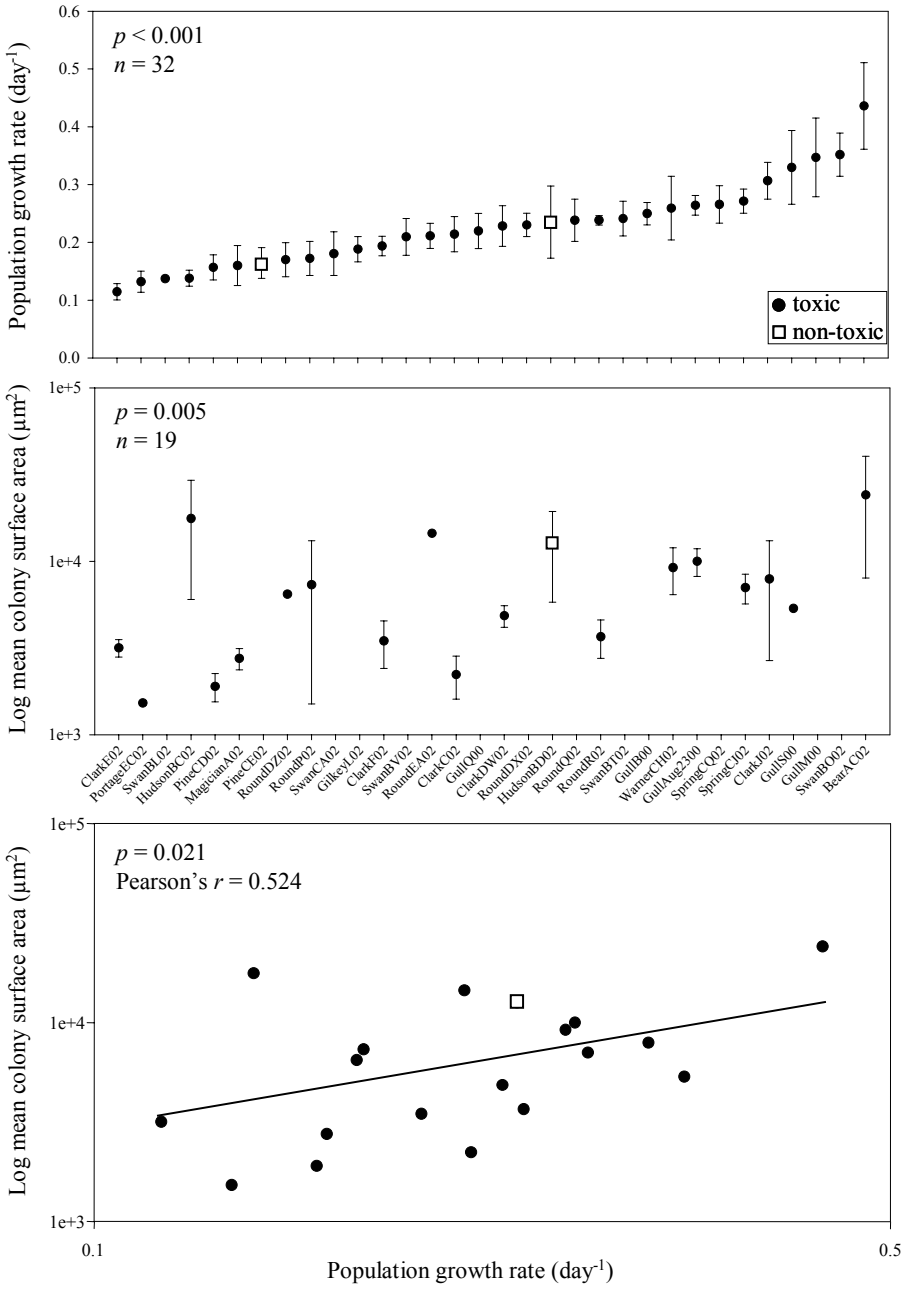

Figure 3.1. Population growth rates (day^{-1}) for 32 *M. aeruginosa* strains from 12 Michigan lakes (top panel). Colony surface area (log surface area (µm^2)) for the 19 of 32 *M. aeruginosa* clones that formed colonies throughout the experiment (middle panel). Results in middle panel exclude one outlier (studentized residual = -3.5); including outlier $p = 0.070$. Relationship between population growth and colony surface area (bottom panel). Filled circles = toxic strains of *M. aeruginosa* [contain toxin gene (*mcyA*) and/or shown to produce microcystins] Empty squares = non-toxic strains of *M. aeruginosa* that lack *mcyA* gene and do not produce microcystins. Error bars = 1 standard error. Note that middle and bottom panels have log-transformed axes.

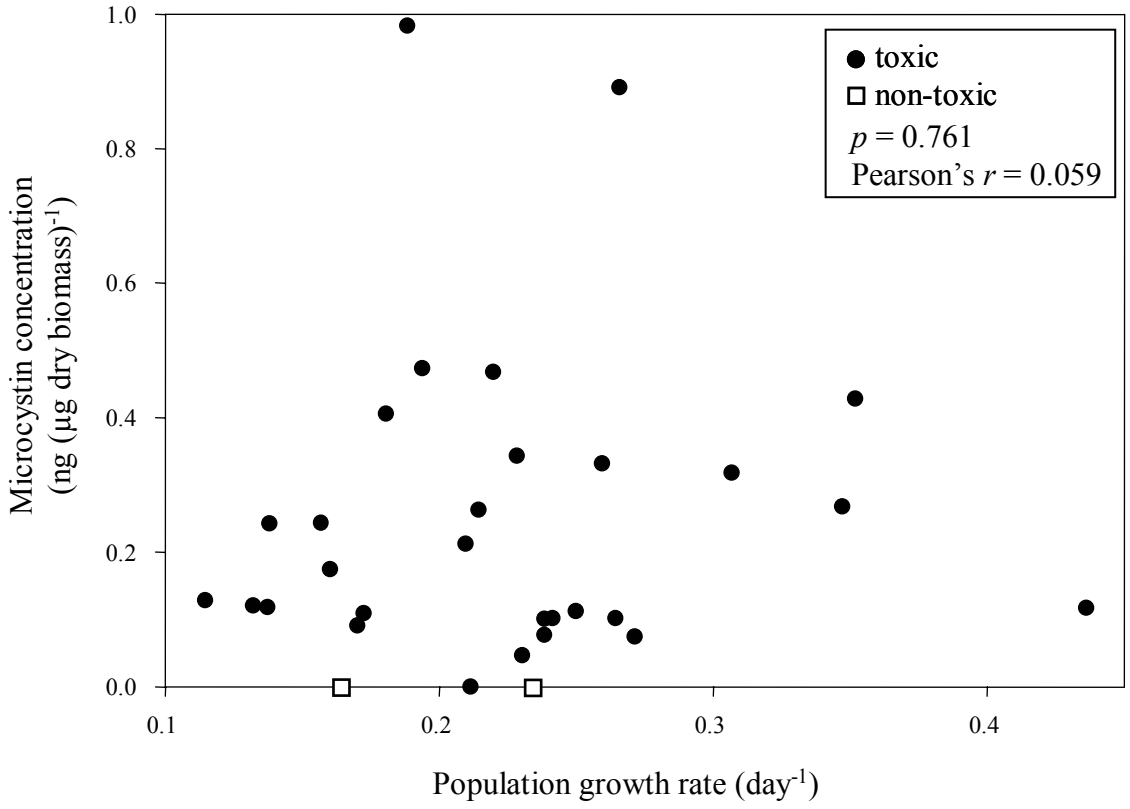

Figure 3.2. Relationship between maximum population growth rate (day^{-1}) and toxin content (ng (µg dry mass)$^{-1}$) for 29 *M. aeruginosa* strains (toxin data not available for GullS02). Results in panel exclude two non-toxic strains. Filled circles = toxic strains of *M. aeruginosa* [contain toxin gene (*mcyA*) and/or shown to produce microcystins]. Empty squares = non-toxic strains of *M. aeruginosa* that lack *mcyA* gene and do not produce microcystins.

Table 3.1. Average growth rates, cell diameters, colony surface areas, and microcystin concentrations (for all toxigenic strains) for *M. aeruginosa* from 12 lakes in the southern peninsula of Michigan. *n* = genetically distinct *M. aeruginosa* strains per lake. SD = standard deviation.

	Growth rate (day^{-1})			Cell diameter (μm)			Colony surface area (μm^2)			Microcystins ($ng\ (\mu g\ dry\ mass)^{-1}$)		
Lake	*n*	mean	SD	*n*	mean	SD	*n*	mean	SD	*n*	mean	SD
Bear	1	0.44	--	1	5.20	--	1	24133	--	1	0.1173	--
Clark	5	0.21	0.07	5	4.76	0.20	5	4327	2215	5	0.3053	0.1253
Gilkey	1	0.19	--	1	4.41	--	0	--	--	1	0.9831	--
Gull	5	0.28	0.05	5	4.60	0.42	2	7673	3285	4	0.2375	0.1713
Hudson	2	0.19	0.07	2	4.78	0.11	2	15132	3597	1	0.2426	--
Magician	1	0.16	--	1	4.70	--	1	2754	--	1	0.1746	--
Pine	2	0.16	0.01	2	4.10	1.40	1	1900	--	1	0.2436	--
Portage	1	0.13	--	1	4.98	--	1	1524	--	1	0.1207	--
Round	6	0.21	0.03	6	4.78	0.15	4	7996	4617	6	0.0709	0.0410
Spring	2	0.27	0.00	2	4.61	0.13	1	7052	--	2	0.4831	0.5773
Swan	5	0.22	0.08	5	4.95	0.26	0	--	--	5	0.2533	0.1554
Warner	1	0.26	--	1	4.63	--	1	9193	--	1	0.3319	--
		$p = 0.034$			$p = 0.574$			$p = 0.013$			$p = 0.054$	

Table 3.2. Results from correlation analysis for *M. aeruginosa* growth rate, cell size, colony surface area, toxin content, and several limnological parameters of the lakes from which strains were collected. *n* = sample size. Pearson's *r* = Pearson's product moment correlation coefficient. * = toxin content for GullS02 not determined.

	Growth rate (day^{-1})			Cell diameter (µm)			Colony surface area ($µm^2$)		
Variables	*n*	Pearson's *r*	*p*-value	*n*	Pearson's *r*	*p*-value	*n*	Pearson's *r*	*p*-value
Cell diameter (µm)	32	0.061	0.741	- -	- -	- -	- -	- -	- -
Colony surface area ($µm^2$)	19	0.524	0.021	19	0.151	0.538	- -	- -	- -
Toxin (ng (µg dry biomass)$^{-1}$)*	29	0.059	0.761	29	-0.219	0.255	17	-0.226	0.382
Total phosphorus (µg L^{-1})	32	0.150	0.412	32	0.237	0.192	19	0.460	0.047
Lake chlorophyll (µg L^{-1})	32	0.051	0.783	32	0.286	0.112	19	0.691	0.001

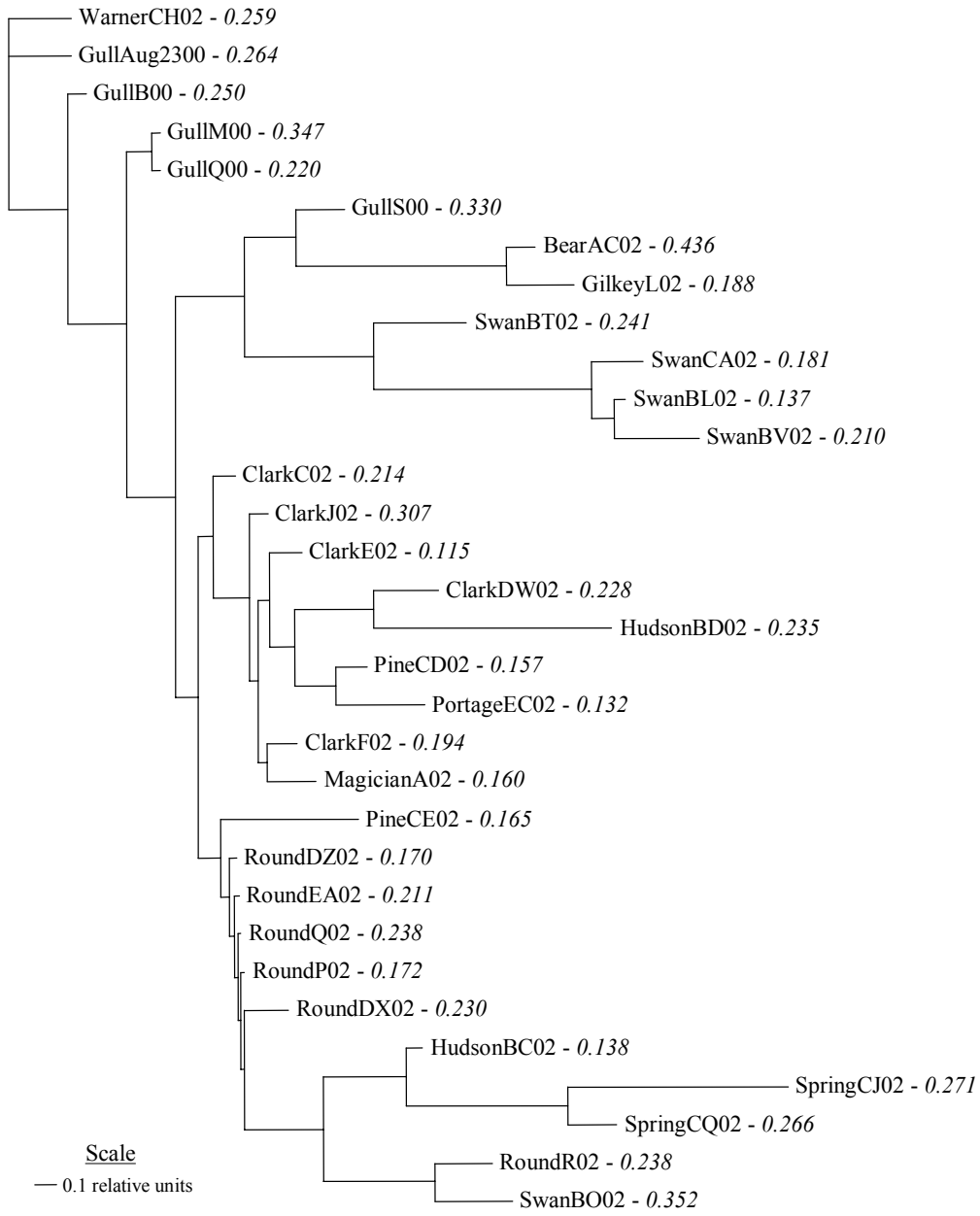

Figure 3.3. Phylogenetic tree for *M. aeruginosa* isolates created by calculating the relative "distances" of PCR products in a pair-wise fashion between the banding patterns of each HIP PCR (see Wilson et al. 2005 for more details about the genetic and phylogenetic analyses). Growth rate estimates are provided next to each clone name in italics. Scale = 1 relative distance unit.

Discussion

We documented significant variation in the maximum population growth rate and in morphological characteristics of thirty-two strains of *M. aeruginosa* (Fig. 3.1) collected from 12 lakes in Michigan. Growth rates and colony sizes differed significantly among both genotypes and lakes (Table 3.1), however we did find similar growth rates for related *M. aeruginosa* strains (Fig. 3.3). The growth rates observed in this study (range = 0.12 to 0.44 day^{-1}) were similar, if somewhat low relative to rates in previous studies in which *Microcystis* was grown under similar environmental conditions (range = 0.05 to 1.11 day^{-1}; Robarts and Zohary 1987; Hesse and Kohl 2001; Hesse et al. 2001; Oh et al. 2000; Long et al. 2001). However, many of the past studies that measured growth rate of *Microcystis* in addition to other traits, like carbon fixation and microcystin production, incorporated single-celled culture collection strains of *Microcystis.* Although we found that single-celled and colonial strains exhibited similar growth rates in the laboratory ($p = 0.710$), extrapolating the results of the previously described studies to effects on colonial forms of *Microcystis*, the morphology observed in nature (Komárek 1991), could be problematic given that cyanobacterial morphology may affect nutrient uptake rates, buoyancy regulation, and vulnerability to grazing (Reynold 1984). An alternative for future research could simply be to focus on understanding the effects of environmental manipulations directly on the diagnostic forms of cyanobacteria observed in nature (e.g., *Microcystis* = colonies), because it is unclear whether strains whose morphology has been altered by culture conditions have not also been affected in other ways (e.g., physiological or phenotypic traits). Cell diameters and microcystin

concentrations were also within the range of previous estimates found for *Microcystis* (Rohrlack et al. 2001b; Vézie et al. 2002).

Interestingly, there was a significant positive correlation between maximum population growth rate and average colony surface area for the colonial strains of *M. aeruginosa* (Fig. 3.1, Table 3.2). This finding is counter-intuitive given that nutrient transfer efficiency predicts that smaller colonies should grow more rapidly than larger colonies due to their greater surface-to-volume ratios (Reynolds 1984; Fogg 1991). Also, larger colonies should suffer more from self-shading than smaller colonies, potentially lowering biomass specific growth rates in large forms. One explanation for the surprising relationship between colony size and growth rate could be that for colonial cyanobacteria, like *Microcystis*, growth occurs via binary fission of individual cells that are held together within a mucus sheath (Komárek 1991; Watanabe et al. 1996). As cells divide, the colony gets larger. Thus, faster growing strains could create larger colonies simply by their intrinsically greater rate of growth. Our results suggest that over the short term (i.e., 9 days), there does not appear to be any growth rate penalty for colonies getting larger, however ecological advantages may exist for large colonial forms. For example, large colonies may be too large for grazers to handle and ingest (Webster and Peters 1978) or have better migratory capabilities which enable large phytoplankters to outcompete other, smaller phytoplankters for light (Reynolds 1984).

Few other significant relationships were found for physiological traits of *M. aeruginosa* and environmental characteristics of the lakes from which the strains were isolated (Table 3.2); including positive correlations between colony surface area and lake total phosphorus (Table 3.2, Pearson's $r = 0.460$, $p = 0.047$) and chlorophyll

concentrations (Table 3.2, Pearson's $r = 0.691$,$p = 0.001$). Based on 12 comparisons (Table 3.2), less than one significant relationship would be expected by chance ($\alpha < 0.05$), however we might expect lake productivity (measured as chlorophyll *a* or phosphorus concentrations) to be related to certain physiological characteristics of cyanobacteria, including growth rate or morphology, given that lake nutrients levels could select for specific strains of *Microcystis* that can exploit *in vitro* nutrient levels similar to those observed in their natural habitat (Wilson et al. 2005). We hypothesized that *M. aeruginosa* strains from high-nutrient lakes would have higher maximum (i.e., nutrient saturated) population growth rates since this would enable them to exploit generally higher levels of available nutrients. However, we did not find a significant correlation between growth rate and lake productivity ($p \geq 0.412$; Table 2) for 32 *M. aeruginosa* strains isolated from 12 lakes that varied in phosphorus and chlorophyll *a* content. We are not aware of other studies that have attempted to correlate phytoplankter traits to the collection sites across so many conspecific strains of cyanobacteria. Our results show that significant relationships exist between the colony size of distinct *M. aeruginosa* and productivity of the habitats where the strains were collected (Fig. 3.1, Table 3.2), however future studies might want to extend our findings to recently isolated *Microcystis* strains from different habitat types or other different cyanobacteria genera, in general. Such comparisons could have important implications on our current knowledge of the ecological drivers of harmful algal bloom promotion or control.

Microcystins are hepatotoxic, secondary metabolites produced by several cyanobacterial genera and are considered to be toxic to grazers (Lampert 1987). Optimal defense theory (Rhoades 1979; Herms and Mattson 1992) suggests that prey have finite

resources to devote towards growth, defense, and maintenance, so allocations to defenses should require investment and opportunity costs that will slow growth (Cronin 2001). Thus, prey can either grow fast while having poor defenses (i.e., produce little or no microcystins) or grow slowly and have better defenses (i.e., produce much microcystins). If microcystins are reliable, but energetically costly, chemical defenses produced by some *Microcystis* strains (Wilson et al. 2005), we would expect that strains producing low levels of microcystins would tend to grow more rapidly in the absence of consumers, while strains producing high concentrations of microcystins would grow more slowly. We found no such pattern for any of the physiological characteristics of the toxic *M. aeruginosa* strains used in this study given that non-toxic strains of *M. aeruginosa* exhibited growth rates and morphological characteristics within the range of the toxic strains (Table 3.2, Fig. 3.2). However, our sample size of non-toxic strains was very small ($n = 2$) relative to toxigenic strains ($n = 30$), but there was variance within the toxic *M. aeruginosa* strains included in our analysis, thus correlations could be tested. No significant correlations were found for toxin content and physiological traits of the toxic *M. aeruginosa* strains (Table 3.2). Thus, no cost of microcystin production was apparent in our study, which is consistent with at least one other study which did not find a difference in maximum population growth rate for a toxic and a non-toxic strain of *M. aeruginosa* (Rohrlack et al. 2001a). However, a cost to toxin production may exist, but we may have not measured the proper response variable, such as nutrient content, nutrient uptake rates, and palatability. An alternative explanation could be that microcystins may play other ecological roles unrelated to defense.

Large genetically-based trait variation within species could influence competitive and trophic interactions by introducing variance in how a species interacts with its consumers and competitors. Thus, future theoretical and empirical studies might consider extending our observations to evaluate the ecological and evolutionary consequences of intraspecific variation of harmful bloom-forming cyanobacteria on community and ecosystem dynamics, given that such variation could help explain the dominance of cyanobacteria over other phytoplankters (Hutchinson 1961; Porter 1977).

CHAPTER 4

VARIANCE IN THE EFFECT OF A CYANOTOXIN AGAINST *DAPHNIA PULICARIA*: A DIRECT TEST OF CYANOBACTERIAL CHEMICAL DEFENSE

Abstract

Laboratory experiments lasting 12 days were conducted to determine the effect of the hepatotoxic, cyclic peptide, microcystin-LR and the lipophilic extract of a toxic *Microcystis aeruginosa* strain on the somatic and population growth rates of two *Daphnia pulicaria* genotypes. The two *Daphnia* clones showed contrasting responses to a diet containing 80% live *Chlorella* and 20% live *Microcystis* (diet containing 535 μg microcystin (g dry weight)$^{-1}$). Both experiments used diets composed of freeze-dried *Chlorella* treated with solvents (control diets), treated with solvents containing microcystin-LR so as to achieve 42 μg of microcystin-LR (g dry weight^{-1}) or treated with solvents and a crude lipid soluble extract of *Microcystis* (no microcystins). Diet type did not affect somatic growth of either *Daphnia* clone on any of the diets containing freeze-dried *Chlorella*. However, diet type did affect population growth of one of the two *Daphnia* clones in both experiments. Surprisingly, the clone that grew best on the diet containing live *Microcystis* was suppressed by the diet containing particle-bound microcystin, while the *Daphnia* clone that grew poorly on live *Microcystis* was not. Both *Daphnia* clones showed increased fecundity on a diet supplemented with the crude lipophilic extract of a toxic *Microcystis* strain, however this translated into enhanced population growth for only one of the *Daphnia* strains due to diet affected survivorship

for the other *Daphnia* clone. Our results demonstrate that a toxic strain of *Microcystis* is not defended by lipophilic secondary metabolites, but that the water-soluble hepatotoxin, microcystin-LR, can negatively affect herbivore population growth in isolation from other co-varying factors, such as nutritional deficiencies or morphology. However, this effect is not universal, and care should be taken before generalizing the effects of specific cyanobacterial compounds across grazer genera, species, or genotypes.

Introduction

Freshwater and estuarine blooms of cyanobacteria threaten global water supplies (Paerl 1988; Morris 1999), because they produce toxins that can harm or kill fishes, livestock, and humans (Carmichael 1992; Chorus et al. 2000; Carmichael et al. 2001). Some of the more commonly studied cyanotoxins include cyclic peptides that target mammalian livers (e.g., microcystins and cylindrospermopsin), nerve synapses (e.g., anatoxin-*a*), and gastrointestinal tracts (e.g., lyngbyatoxin-*a*) (Carmichael 1992; Chorus and Bartram 1999; Zurawell et al. 2005). The effect of these compounds on ecologically relevant organisms, such as herbivorous zooplankton, is equivocal (Wilson et al. in press), despite a large literature that indirectly suggests effects of cyanotoxins on freshwater zooplankton (Lampert 1987; Zurawell et al. 2005).

Cyanobacteria have been proposed to lower zooplankton grazing rates in three basic ways: (1) by occurring as large colonial and filamentous morphologies that clog filtering appendages or are otherwise inedible, (2) by being nutritionally deficient, and (3) by producing toxic secondary metabolites (Porter and Orcutt 1980; Lampert 1987; DeMott 1989). There are direct tests of the effects of morphology (Ferrão-Filho and Azevedo 2003) and nutrition (DeMott and Müller-Navarra 1997; von Elert and Wolffrom 2001; Ravet et al. 2003) on zooplankton feeding, but no study has unambiguously tested the effect of cyanobacterial toxins on zooplankton fitness. Past experiments have compared zooplankton performance when (1) immersed in media containing dissolved cyanotoxins (Gilbert 1990; DeMott et al. 1991; Reinikainen et al. 2001; Wiegand et al. 2002) or cyanobacterial extracts (Wheeler et al. 1942; Shelubsky 1951; Stangenburg 1968), (2) fed diets containing cyanobacteria versus being starved (Arnold 1971; Lampert

1981a,b; reviewed by Wilson et al. in press), (3) fed diets containing cyanobacteria versus foods supporting better growth (Arnold 1971; Lürling 2003a; reviewed by Wilson et al. in press), (4) fed diets of two conspecific cyanobacteria that are or are not toxigenic to mammals (Henning et al. 1991; Smith and Gilbert 1995), and (5) fed diets consisting of a wild-type cyanobacteria containing microcystins or its mutant which lacks the ability to produce microcystins (Rohrlack et al. 1999a; Kaebernick et al. 2001; Rohrlack et al. 2005). Results from these types of studies show that cyanobacteria are poor food for grazers, but cannot unambiguously demonstrate the effects of consuming (as opposed being immersed in) cyanobacterial secondary metabolites (see review by Wilson et al. in press), because cyanotoxins are either present in unrealistic forms (dissolved in water rather than bound in cells) or are potentially confounded by other factors (i.e., species or strains with and without the toxin may differ in other respects, as well). Experiments where grazers are immersed in media containing secondary metabolites do not mimic the typical route of exposure for zooplankton (i.e., grazing; DeMott and Dhawale 1995) and routinely use toxin concentrations several orders of magnitude higher (DeMott et al. 1991) than found in nature (Christoffersen 1996; Chorus and Bartram 1999). Furthermore, a recent meta-analysis of the zooplankton-cyanobacteria provides equivocal support for the role of known toxic compounds on zooplankton (Wilson et al. in press).

To test directly for negative effects of the hepatotoxic, cyclic peptide, microcystin-LR, we used an experimental technique, routinely used to identify chemical defenses of marine macroalgae (i.e., freeze-dried seaweeds coated with solvent and/or algal secondary chemistry; Hay et al. 1998) but never for dried phytoplankton (but see Ianoro et al. 2004 for a similar experimental design using live phytoplankton). To see if

lipid-soluble compounds from a microcystin-containing strain of *Microcystis* might also be affecting consumers, we conducted a parallel experiment assessing the effects of the crude lipophilic extract from this alga. We tested effects against two clones of the lake-dwelling cladoceran *Daphnia pulicaria* that vary in their response to a diet containing live toxic *Microcystis* (Sarnelle and Wilson 2005). Freeze-dried algae have never been used for these types of experiments, which is surprising given that several studies show that zooplankton perform well on dried algae (Naylor et al. 1993; Navarro 1999; García et al. 2003; but see Dobberful and Elser 1999 for effects of freeze-dried algae vs. oven-dried algae on *Daphnia magna* fitness). Drying phytoplankton can alter certain nutrient and biochemical constituents, like phosphorus, nitrogen, and protein content, however other important components, like carbon and lipid content, may remain unchanged (Dobberfuhl and Elser 1999). Also, morphology remains relatively unaffected after drying and rehydration in zooplankton media for certain phytoplankters (e.g., round chlorophytes, *Chlorella*), however others can change drastically (e.g., needle-shaped chlorophytes, *Ankistrodesmus*). Freeze-dried algae make realistic experimental foods for the types of comparisons described in this study given that morphology and nutritional content are consistent for treatment and control foods (Hay et al. 1998), thus the only difference between the diets is the chemistry of interest. Furthermore, this type of experimental technique is especially suited for unselective grazers, like *Daphnia*; in contrast, more selective grazers like copepods (DeMott 1986; Engström et al. 2000; Becker et al. 2004) may ignore dead phytoplankton-based foods.

Methods

Daphnia collection

One *Daphnia pulicaria* female was isolated from each of two small (< 30 ha) glacial lakes in southern Michigan in 2004. Descendents from these mothers were used in this study. The Lake Sixteen clone (SI4) isolate originated from oligotrophic (summer mixed layer total phosphorus concentration 9-12 μg L^{-1}; Sarnelle and Wilson 2005) Lake Sixteen (latitude north 42°33'90", longitude west 85°36'80"). The Baker Lake clone (BA2) isolate originated from meso-eutrophic (summer mixed layer total phosphorus concentration 20-41 μg L^{-1}; Sarnelle and Wilson 2005) Baker Lake (latitude north 42°38'90", longitude west 85°30'20"). Long-term quantitative phytoplankton data do not exist for these lakes, but observations by the authors and others (C. Cáceres, S. Hamilton, and A. Tessier, personal observations) suggest that Baker Lake experiences cyanobacterial blooms (e.g., *Microcystis aeruginosa* was abundant in the lake when we collected *D. pulicaria*). In contrast, using microscopy, we observed no evidence of cyanobacteria in Lake Sixteen when we collected the *Daphnia*. Consistent with this single observation, an existing empirical model using data from Michigan lakes (Raikow et al. 2004) estimates that, based on summer epilimnion phosphorus concentrations, cyanobacteria should be abundant in Baker Lake (69% of total phytoplankton biovolume) but relatively rare (<9%) in Lake Sixteen. Recent studies show that the introduction of zebra mussels (*Dreissena polymorpha)*, can cause cyanobacterial blooms in phosphorus poor lakes (Vanderploeg et al. 2001; Raikow et al. 2004; Sarnelle et al. 2005; Knoll et al. in press), but zebra mussels had not invaded either lake at the time of *Daphnia* collection.

***Daphnia* genetic analysis**

The 2 *Daphnia* isolates were genetically discriminated using variation of three microsatellite loci (Dp464, Dp496, Dp502; Colbourne et al. 2004). Multiple live animals from each clone were rinsed thoroughly with distilled water to remove attached bacteria and phytoplankton, and then genomic DNA was extracted with a Qiagen DNeasy kit following the manufacturer's protocol. Amplification of microsatellite alleles via PCR was performed in 10ul volumes (10-50 ngDNA, 1 µl 10x buffer [10mM Tris HCl at pH 8.3, 50mM KCl, 0.001% gelatin, and 1.5mM $MgCl_2$], 0.2 µl dNTPs, 0.15 µl forward primer with attached fluorescent label, 0.15 µl reverse primer, and 1 unit of Taq DNA polymerase). Each locus was separately analyzed for each *Daphnia* sample (6 reactions). PCR was performed on a Robocycler 40 gradient under the following conditions: 95°C for 2 min followed by 39 cycles of 95°C for 30 s, 55°C for 20 s, and 72°C for 30 s with a final extension at 72°C for 7 min. PCR products were analyzed using an ABI Prism 3100 genetic analyzer and microsatellite nucleotide lengths were determined with Genescan 3.7 and Genotyper 3.7 software (Applied Biosystems).

The two *Daphnia* exhibited different nucleotide lengths for two of the microsatellite primers (Dp464 [Lake Sixteen clone = 148, 148; Baker Lake clone = 147, 147] and Dp496 [Lake Sixteen clone = 196, 202; Baker Lake clone = 201, 216]), but not Dp502 [149, 149]. The *Daphnia* also exhibit subtle phenotypic differences in culture. For example, the Baker Lake clone is darker than the Lake Sixteen clone. Moreover, the Lake Sixteen clone tends to produce dark ephippia in old, dense cultures while the Baker Lake clone only produces light-colored ephippia. Finally, a recent study demonstrated that the somatic growth of the Baker Lake clone was less inhibited ($p = 0.002$) by a diet

comprised entirely of a toxic *Microcystis* strain than was the Lake Sixteen clone (Sarnelle and Wilson 2005).

Daphnia maintenance and experimental design

Daphnia clones were maintained in a temperature controlled room (25°C) in lake water (Lake Lanier, oligotrophic lake in north Georgia) on a mixture of green algae, including *Ankistrodesmus*, *Chlorella*, and *Chlamydomonas* grown in a nutrient-rich medium (modified BG-11 medium, Vanderploeg et al. 2001) for many generations prior to the start of the experiments.

Three experiments were performed to determine the effect of (1) a live cyanobacterium, (2) the cyanotoxin, microcystin-LR, and (3) the crude lipophilic extract of *Microcystis aeruginosa* (UTEX 2667), on the somatic and population growth rates of both clones of *Daphnia*. To test the effect of the presence of toxic *Microcystis* on *Daphnia* growth, *Daphnia* were fed (1) *Chlorella* by itself or (2) a mixture of 80% *Chlorella* and 20% *Microcystis aeruginosa* UTEX 2667 by biovolume. The *Chlorella* strain used in all of the experiments originated from Zaca Lake, California (O. Sarnelle, *personal communication*), was unicellular, averaged 5-12 μm in diameter, and is nutritionally sufficient for the long-term maintenance of *Daphnia* cultures in our laboratory. *Microcystis* UTEX 2667 grows only as single-cells (average = 3-5 μm in diameter) and contains microcystins, which are hepatotoxic, phosphatase inhibitors (Carmichael 1992). Live algae were prepared by growing cells in batch cultures using a nutrient rich medium (modified BG-11 medium, Vanderploeg et al. 2001), concentrating live, exponentially growing cells in a centrifuge, and then resuspending the algal pellet in filtered ($\leq$ 0.7 μm) lake water.

To test the effect of microcystin-LR on *Daphnia* growth, *Daphnia* were fed (1) freeze-dried *Chlorella* treated with dimethyl sulfoxide (DMSO) or (2) freeze-dried *Chlorella* treated with DMSO into which we had dissolved microcystin-LR. In both cases, the DMSO was removed via rotary evaporation and cells were stored frozen under nitrogen until use. Freeze-dried *Chlorella* was prepared by growing batch cultures of the alga, concentrating exponentially growing cells in a centrifuge, freezing the cells, and then freeze-drying the alga. Physically, freeze-dried cells look very similar to live cells, but are slightly smaller (5-9 μm in diameter). To prepare the diets, a similar amount of freeze-dried *Chlorella* was added to two sets of glass vials and the *Chlorella* was treated with 0.4 ml DMSO (control diet) or a mixture of 0.4 ml of DMSO containing 83 μg of microcystin-LR (treatment diet; toxin purchased from AXXORA, LLC).

We assessed the potential effects of the crude lipid soluble secondary metabolites of *Microcystis* on *Daphnia* growth by feeding (1) freeze-dried *Chlorella* treated with organic solvents or (2) freeze-dried *Chlorella* treated with organic solvents containing the crude lipophilic extract (2x natural concentration based on algal dry weight) of an exponentially growing *Microcystis* UTEX 2667 culture. Freeze-dried *Chlorella* was prepared in the same manner as described above. To prepare the diets in this experiment, a similar amount of dried *Chlorella* was added to two sets of glass vials and the *Chlorella* was treated with 0.4 ml of the organic solvent mixture (1 part methanol: 1 part ethyl acetate: 3 parts ether) (control diet) or a mixture of 0.4 ml of the organic solvent mixture including the lipophilic extract of *Microcystis*. The extract of *Microcystis* UTEX 2667 was prepared by extracting twice the amount (by dry weight) of freeze-dried *Microcystis* cells (dried cells prepared with the identical protocol described above for *Chlorella*) as

the total weight of dried *Chlorella* cells loaded into the experimental vials. We extracted 2x the natural concentration by dry mass to correct for losses due to extraction inefficiencies and compounds lost on the glassware. Dried *Microcystis* cells were extracted under sonication with a mixture of 50% water: 50% methanol (two extractions – one hour per extraction) and a mixture of 25% methanol: 75% ethyl acetate (two extractions – one hour per extraction). The crude extract was passed through a GF/F filter (retains particles ≥ 0.7 μm) to remove particles and a liquid:liquid partition of water:ethyl acetate was used to separate the water soluble from the lipophilic compounds. The water fraction, which would contain the microcystins, was discarded. The solvent in the lipophilic fraction was removed using a speed vacuum concentrator. The dried lipophilic fraction, which dissolved quickly in the organic solvent (1 part methanol: 1 part ethyl acetate: 3 parts ether), was mixed thoroughly in each vial, and then an equal amount of the mixture (0.4 ml) was added to the treatment experimental vials for this experiment. The samples were dried via evaporation using a speed vacuum concentrator and stored under nitrogen in the freezer until use.

The experimental design was similar for all experiments. Each experiment was conducted in a temperature controlled room (25°C) under dim lighting provided by cool, white, fluorescent lights (light 18 hours: dark 6 hours). Prior to the experiments, neonates from the stock cultures of each *Daphnia* clone were placed in separate vials filled with glass-fiber filtered lake water and fed daily with *Chlorella* at concentrations above limiting levels (> 1 mg carbon L^{-1}) until maturity. Neonates (< 24 hours old) from these mothers were pooled and then each experiment was initiated (day 1) by adding five (experiments consisting of live foods or freeze-dried foods treated with organic solvents

and the lipophilic extract of *Microcystis*) or six (experiment consisting of freeze-dried foods treated with DMSO and microcystin-LR) randomly chosen neonates to five replicate 500 ml bottles per treatment (total 60 experimental bottles used for all three experiments for both *Daphnia* clones) filled with filtered lake water (≤ 0.7 μm) and a biovolume of particulate food equivalent to 1 mg carbon L^{-1} (6.5×10^6 μm^3 ml^{-1}; biovolume to carbon conversion from Kilham et al. [1997]). Food concentrations were determined by counting 20 fields of two replicate 0.125 ml sub-samples from each treatment using a Palmer-Maloney chamber. A random subset of neonates from both *Daphnia* clones was collected and measured to estimate the initial lengths at the beginning of each experiment. The bottles were sealed and placed on a rotating plankton wheel (0.5 rotations per minute). *Daphnia* were transferred to new bottles with filtered lake water (all treatments) and food daily, except for treatments used in the first experiment where the grazers were moved to new bottles with fresh media every 2 days. Survivors were transferred to the new bottle, *Daphnia* carrying eggs were identified, and neonates were counted and discarded. On day 5 (experiment consisting of live foods) or 6 (experiments consisting of freeze-dried foods treated with solvents and microcystin or the lipophilic extract of *Microcystis*), all live *Daphnia* were individually placed into a water droplet on a microscope slide and measured with a compound microscope to estimate juvenile somatic growth rates (length μm, day^{-1}) using the formula:

$\frac{\ln L_{t1} - \ln L_{to}}{time}$, where L_{to} and L_{t1} are initial (day 1) and later (day 5 or 6) animal lengths, respectively.

Time to maturity was determined as the time when all surviving *Daphnia* in each bottle carried eggs, since each bottle contained more than one animal and it was

impossible to determine when specific *Daphnia* carried eggs. Females in some of the treatments never carried eggs and we wanted to compare time to maturity across treatments, so we estimated the time for 50% of the mothers to reach maturity (50% egg sighting; ES_{50}). The total number of live neonates produced per mother was calculated by dividing the total number of neonates in each bottle by the number of surviving mothers per day and then summing these values across the entire experiment for each bottle for each day of the experiments. One replicate (with 100% survival on day 9) was spilled on day 10 for the Baker Lake clone (lipophilic extract treatment), so total neonates per mother for 12 days could not be used for this replicate. Population growth rates (r) were iteratively calculated for each beaker using the Euler equation: $1 = \sum_{x=0}^{k} e^{-rx} l(x) m(x)$, where r is the rate of population growth (day^{-1}), x is the age class (day; 0 to 12), $l(x)$ is the probability of surviving to age x, and $m(x)$ is the number of neonates produced per *Daphnia* per bottle on day x. In case of no reproduction, r was determined from changes in abundance over time ($r = \frac{\ln density_{t+1} - \ln density_t}{time}$).

Microcystin concentrations were determined via enzyme-linked immunosorbent assay (ELISA; An and Carmichael 1994). Filtered samples for all treatment diets were collected on pre-weighed GF/F filters from each experiment, dried, weighed to determine dried algal biomass, stored frozen, and subsequently extracted twice in 75% aqueous methanol before ELISA. Filtered (GF/F) whole water samples from the microcystin-LR addition treatment were also analyzed for ELISA to determine the dissolved microcystin concentration in this treatment.

Statistical analyses

Differences among treatment effects were assessed via analysis of variance (ANOVA). Two-way ANOVA assessed interactions between *Daphnia* clone and treatment food types. *T*-tests determined if growth rates were different from 0. Pearson's product moment correlations and Bonferroni adjusted probabilities determined how well somatic growth rates were related to population growth rates. Data were log transformed as needed to conform to the assumptions of parametric statistics. All analyses were performed with Systat 11 (Systat Software, Inc. 2004).

Results

Over 12 days, survivorship was adequate to high for all fed treatments of both clones [Baker Lake mean = 74% survivorship; range = 40% - 100% survivorship; Lake Sixteen mean = 79% survivorship; range 56% -100% survivorship]. Survivorship never differed significantly among any of the fed treatments for all three experiments.

When fed live foods, both *Daphnia* clones showed greater somatic growth and neonate production ($p \leq 0.007$; Fig. 4.1a,b, Tables 4.1 and 4.2), faster maturity (i.e., ES_{50}; $p \leq 0.001$; Fig. 4.2a,b), and higher population growth rate ($p \leq 0.002$; Fig. 4.1c, Table 4.3) on diets of 100% *Chlorella* compared to diets of 80% *Chlorella* and 20% *Microcystis*. Consistent with a past study (Sarnelle and Wilson 2005), the Baker Lake clone showed faster somatic growth on a diet containing cyanobacteria than the Lake Sixteen clone ($p < 0.001$; Fig. 4.1a). This difference in somatic growth rate translated into greater population growth on the cyanobacteria containing diet for the Baker Lake clone versus the Lake Sixteen clone ($p < 0.001$; Fig. 4.1a,c). The Baker Lake population increased significantly on the mixed diet (*t*-test mean = 0, $p < 0.001$; Fig. 4.1c), while the Lake Sixteen population did not (*t*-test mean = 0, $p = 0.395$; Fig. 4.1c). A statistically significant interaction between diet type and *Daphnia* clone was found for population growth rate ($p = 0.039$; Table 4.3) but not for somatic growth rate and neonate production ($p \geq 0.191$; Tables 4.1 and 4.2).

Comparing *Daphnia* performance when fed diets lacking or containing microcystin-LR, we found that rates for somatic growth ($p \geq 0.308$; Fig. 4.1a, Table 4.1), time to maturity (i.e., ES_{50}; $p \geq 0.186$; Fig. 4.2c,d), and neonate production ($p \geq 0.139$; Fig. 4.1b, Table 4.2) did not differ for each *Daphnia* clone. Averaging across these diets,

the Lake Sixteen clone reached maturity 0.5 days faster (Two-way ANOVA, *Daphnia* factor $p < 0.001$; Fig. 4.2c,d insets) and produced more than 3x the live neonates per female than the Baker Lake clone (Two-way ANOVA, *Daphnia* factor $p < 0.001$; Fig. 4.1b, Table 4.2) which translated into significantly higher population growth for the Lake Sixteen clone than the Baker Lake clone (Two-way ANOVA, *Daphnia* factor $p < 0.001$; Fig. 4.1c, Table 4.3). Feeding *Daphnia* diets treated with microcystin-LR significantly lowered population growth for the Baker Lake clone ($p = 0.032$; Fig. 4.1c, Table 4.3), but not the clone from Lake Sixteen ($p = 0.375$; Fig. 4.1c, Table 4.3). Population growth rates for the Lake Sixteen clone on both treatment types were significantly > 0 (*t*-test, $p \leq 0.001$; Fig. 4.1c), but did not differ from 0 for the Baker Lake clone fed foods treated with DMSO only (*t*-test, $p = 0.563$; Fig. 4.1c). In contrast, population growth rate for the Baker Lake clone fed foods treated with DMSO and microcystin-LR was significantly below 0 (*t*-test, $p = 0.047$; Fig. 4.1c). A statistically significant interaction between diet type and *Daphnia* clone was found for population growth rate ($p = 0.014$; Table 4.3) but not for somatic growth rate and neonate production ($p \geq 0.312$; Tables 4.1 and 4.2).

For *Daphnia* fed freeze-dried *Chlorella* treated with organic solvents or organic solvents supplemented with the lipophilic extract of *Microcystis*, somatic growth rates did not differ within each *Daphnia* clone ($p \geq 0.168$; Fig. 4.1a, Table 4.1). The Baker Lake clone reached maturity faster when fed lipid supplemented food than grazers fed solvent control food ($p = 0.015$; Fig. 4.2c), however the Lake Sixteen clone exhibited similar maturity rates on both food types ($p = 0.277$; Fig. 4.2d). Adding the lipid-soluble extract of *Microcystis* to diets significantly enhanced neonate production of both clones ($p \leq 0.049$; Fig. 4.1b, Table 4.2). The Lake Sixteen clone produced 1.7x more neonates per

female than the Baker Lake clone averaged across both treatment types (Two-way ANOVA, *Daphnia* factor $p < 0.001$; Fig. 4.1b, Table 4.2). The enhanced fecundity of *Daphnia* fed foods supplemented with cyanobacterial lipids translated into higher population growth for the Lake Sixteen clone ($p = 0.002$; Fig. 4.1c, Table 4.3), but not for the Baker Lake clone ($p = 0.291$; Fig. 4.1c, Table 4.3) when compared to population growth on foods treated with organic solvent alone. Population growth rates were 3.9x higher for the Lake Sixteen clone than the Baker Lake clone averaged across both treatment diets (Two-way ANOVA, *Daphnia* factor $p < 0.001$; Fig. 4.1c, Table 4.3). A statistically significant interaction between diet type and *Daphnia* clone was found for population growth rate ($p = 0.025$; Table 4.3) but not for somatic growth rate and neonate production ($p \geq 0.052$; Tables 4.1 and 4.2).

Correlations between somatic and population growth rates across all studies were positive for both *Daphnia* (Pearson $r = 0.551$, $p < 0.001$, $n = 60$) or each *Daphnia* analyzed independently (Baker Lake clone Pearson $r = 0.748$, $p < 0.001$, $n = 30$; Lake Sixteen clone Pearson $r = 0.489$, $p = 0.006$, $n = 30$) and for all treatments and *Daphnia* clones used in the experiments incorporating live foods (Pearson $r = 0.879$, $p < 0.001$, $n = 20$) and foods treated with organic solvents or organic solvents and the lipophilic extract of *Microcystis* (Pearson $r = 0.763$, $p < 0.001$, $n = 20$), but not for foods treated with DMSO or DMSO and microcystin-LR (Pearson $r = -0.051$, $p = 0.832$, $n = 20$).

Cell-bound microcystins were present in the diets containing live *Microcystis* (535 μg (g dry weight)$^{-1}$) and the addition of DMSO with microcystin-LR (42 μg (g dry weight)$^{-1}$). The concentration of microcystin-LR in the latter diet was ≈ 1% of the amount expected to be present (4400 μg (g dry weight)$^{-1}$), thus much of the microcystin-

LR was dissolved in the treatment water (11.9 μg L^{-1}) and not on or in the freeze-dried algae. Microcystins were not detected in the other treatment foods, including the diet containing the lipophilic extract of *Microcystis*.

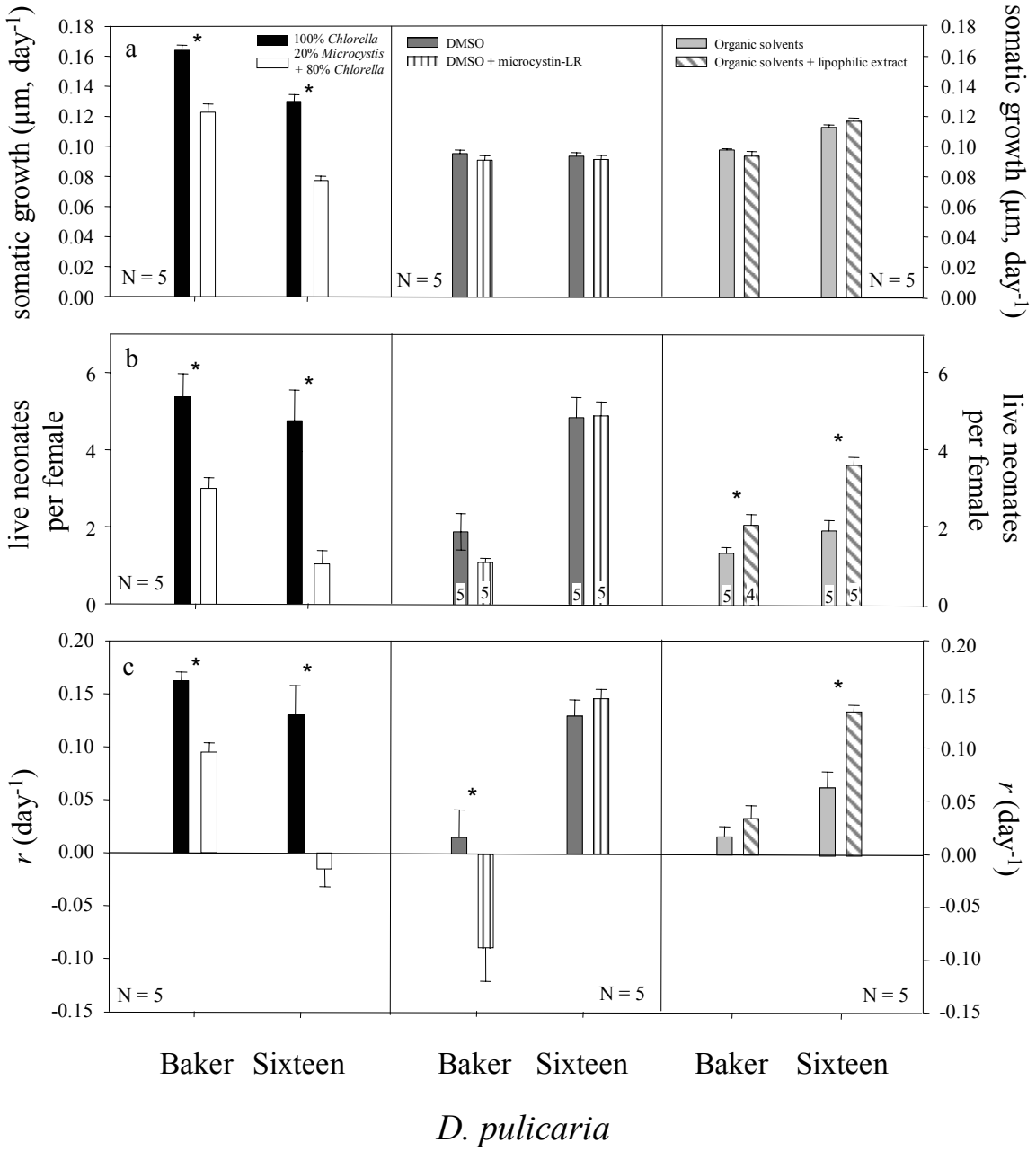

Figure 4.1. Somatic growth rates over 5 or 6 days (4.1a; length µm, day^{-1}), number of live neonates per *Daphnia* mother over 12 days (4.1b), and population growth rates over 12 days (4.1c; *r*, day^{-1}) for two *Daphnia* clones (Baker and Sixteen) from three experiments lasting 12 days each. Experiments included diets composed of 100% *Chlorella* or 20% *Microcystis* and 80% *Chlorella* or composed of freeze-dried *Chlorella* coated with DMSO versus DMSO and microcystin-LR or organic solvents versus organic solvents and the crude lipophilic extract of *Microcystis* UTEX 2667, respectively. * = $p < 0.05$. Error bars = 1 SE. *n* = sample size per treatment per *Daphnia* clone. On day 10, one replicate was spilled for Baker Lake clone of the lipophilic treatment.

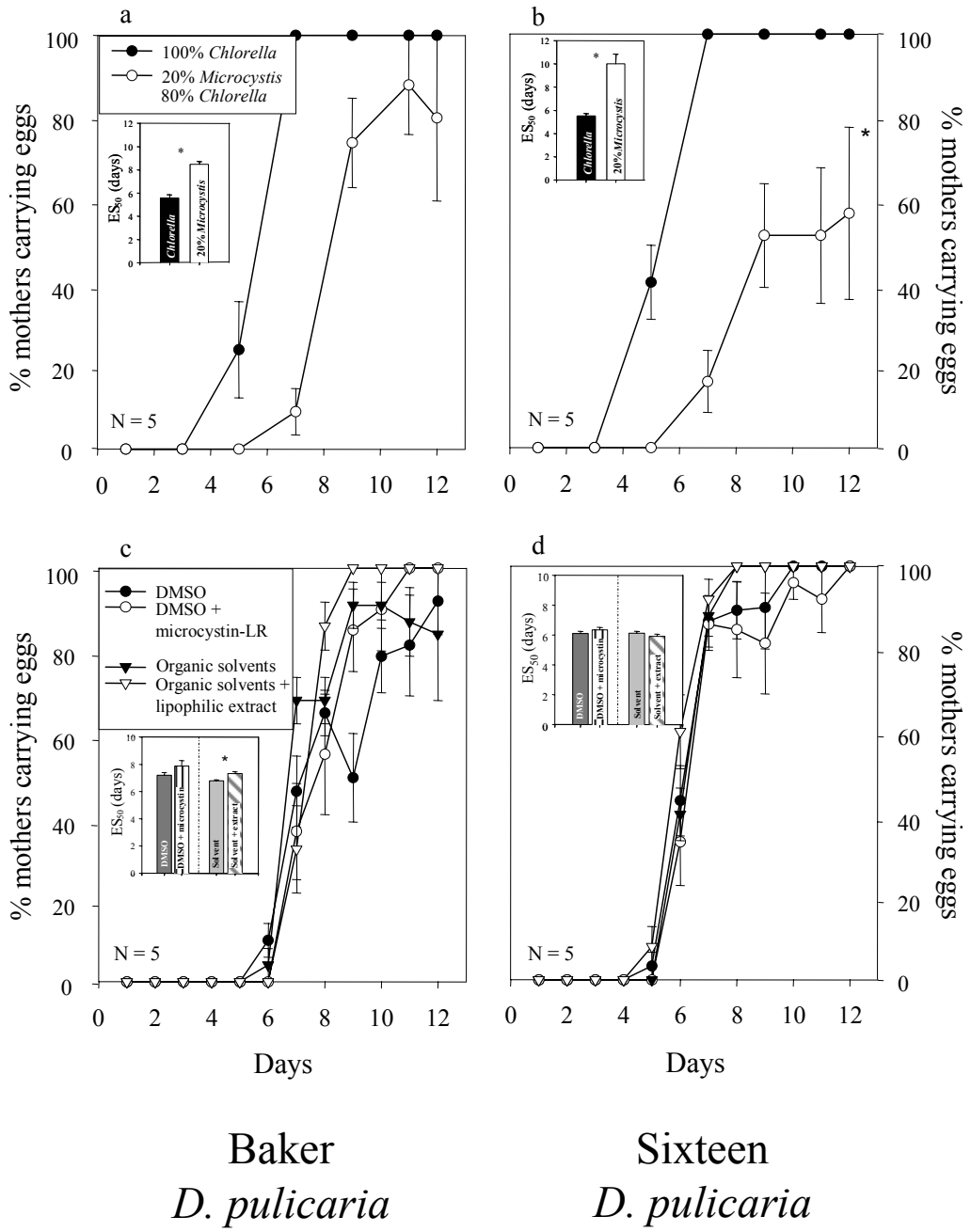

Figure 4.2. Time to maturity (i.e., egg sighting) curves for two *Daphnia* clones (Baker (4.2a,c) and Sixteen (4.2b,d)) from three experiments lasting 12 days each. Experiments included diets composed of 100% *Chlorella* or 20% *Microcystis* and 80% *Chlorella* (4.2a,b) or composed of freeze-dried *Chlorella* coated with DMSO versus DMSO and microcystin-LR (4.2c,d) or organic solvents versus organic solvents and the lipophilic extract of *Microcystis* UTEX 2667 (4.2c,d), respectively. Inset figures provide mean time for 50% of the mothers to reach maturity (ES_{50}). * = $p < 0.05$. Error bars = 1 SE. *n* = sample size per treatment per *Daphnia*.

Table 4.1. Analyses of variance results for somatic growth rate (length μm, day^{-1}) for two *Daphnia* clones used in three experiments. df = degrees of freedom; MS = mean square error.

Experiment	Test	*Daphnia*	Trait	Source	df	MS	F-ratio	*p*-value
100% *Chlorella*,	1-way	Baker 2	Juvenile	Food type	1	0.004	39.953	< 0.001
20% *Microcystis* and	ANOVA		growth rate	Error	8	0.000		
80% *Chlorella*		Sixteen 4		Food type	1	0.007	103.001	< 0.001
				Error	8	0.000		
	2-way	both clones	Juvenile	Food type	1	0.011	126.842	< 0.001
	ANOVA		growth rate	*Daphnia*	1	0.008	92.171	< 0.001
				Interaction	1	0.000	1.863	0.191
				Error	16	0.000		
DMSO,	1-way	Baker 2	Juvenile	Food type	1	0.000	1.183	0.308
DMSO + microcystin-LR	ANOVA		growth rate	Error	8	0.000		
		Sixteen 4		Food type	1	0.000	0.359	0.566
				Error	8	0.000		
	2-way	both clones	Juvenile	Food type	1	0.000	1.462	0.244
	ANOVA		growth rate	*Daphnia*	1	0.000	0.036	0.851
				Interaction	1	0.000	0.166	0.689
				Error	16	0.000		
Organic solvents,	1-way	Baker 2	Juvenile	Food type	1	0.000	1.521	0.252
organic solvents +	ANOVA		growth rate	Error	8	0.000		
lipophilic extract		Sixteen 4		Food type	1	0.000	2.304	0.168
				Error	8	0.000		
	2-way	both clones	Juvenile	Food type	1	0.000	0.003	0.954
	ANOVA		growth rate	*Daphnia*	1	0.002	83.149	< 0.001
				Interaction	1	0.000	3.708	0.072
				Error	16	0.000		

Table 4.2. Analyses of variance results for the number of live neonates produced per mother (over 12 days) for two *Daphnia* clones used in three experiments. df = degrees of freedom; MS = mean square error.

Experiment	Test	*Daphnia*	Trait	Source	df	MS	F-ratio	*p*-value
100% *Chlorella*,	1-way	Baker 2	# of live neonates	Food type	1	14.042	12.901	0.007
20% *Microcystis* and	ANOVA		produced per	Error	8	1.089		
80% *Chlorella*		Sixteen 4	mother	Food type	1	34.534	18.787	0.002
				Error	8	1.838		
	2-way	both clones	# of live neonates	Food type	1	46.309	31.646	< 0.001
	ANOVA		produced per	*Daphnia*	1	8.364	5.715	0.029
			mother	Interaction	1	2.267	1.549	0.231
				Error	16	1.463		
DMSO,	1-way	Baker 2	# of live neonates	Food type	1	1.163	2.700	0.139
DMSO + microcystin-LR	ANOVA		produced per	Error	8	0.598		
		Sixteen 4	mother	Food type	1	0.003	0.003	0.956
				Error	8	1.019		
	2-way	both clones	# of live neonates	Food type	1	0.735	0.909	0.355
	ANOVA		produced per	*Daphnia*	1	57.800	71.515	< 0.001
			mother	Interaction	1	0.882	1.091	0.312
				Error	16	0.808		
Organic solvents,	1-way	Baker 2	# of live neonates	Food type	1	1.165	5.639	0.049
organic solvents +	ANOVA		produced per	Error	7	0.207		
lipophilic extract		Sixteen 4	mother	Food type	1	7.310	24.510	0.001
				Error	8	0.298		
	2-way	both clones	# of live neonates	Food type	1	6.971	27.283	< 0.001
	ANOVA		produced per	*Daphnia*	1	5.519	21.599	< 0.001
			mother	Interaction	1	1.143	4.475	0.052
				Error	15	0.256		

Table 4.3. Analyses of variance results for population growth (r, day^{-1}) for two *Daphnia* clones used in three experiments. df = degrees of freedom; MS = mean square error.

Experiment	Test	*Daphnia*	Trait	Source	df	MS	F-ratio	*p*-value
100% *Chlorella*,	1-way	Baker 2	*r* (intrinsic rate of	Food type	1	0.011	30.969	0.001
20% *Microcystis* and	ANOVA		natural increase)	Error	8	0.000		
80% *Chlorella*		Sixteen 4		Food type	1	0.053	19.761	0.002
				Error	8	0.003		
	2-way	both clones	*r* (intrinsic rate of	Food type	1	0.057	37.119	< 0.001
	ANOVA		natural increase)	*Daphnia*	1	0.026	16.973	0.001
				Interaction	1	0.008	5.072	0.039
				Error	16	0.002		
DMSO,	1-way	Baker 2	*r* (intrinsic rate of	Food type	1	0.027	6.689	0.032
DMSO + microcystin-LR	ANOVA		natural increase)	Error	8	0.004		
		Sixteen 4		Food type	1	0.001	0.883	0.375
				Error	8	0.001		
	2-way	both clones	*r* (intrinsic rate of	Food type	1	0.010	4.026	0.062
	ANOVA		natural increase)	*Daphnia*	1	0.152	63.120	< 0.001
				Interaction	1	0.018	7.548	0.014
				Error	16	0.002		
Organic solvents,	1-way	Baker 2	*r* (intrinsic rate of	Food type	1	0.001	1.277	0.291
organic solvents +	ANOVA		natural increase)	Error	8	0.001		
lipophilic extract		Sixteen 4		Food type	1	0.013	19.977	0.002
				Error	8	0.001		
	2-way	both clones	*r* (intrinsic rate of	Food type	1	0.010	16.156	0.001
	ANOVA		natural increase)	*Daphnia*	1	0.028	45.407	< 0.001
				Interaction	1	0.004	6.067	0.025
				Error	16	0.001		

Discussion

We show that natural levels of particle-bound and dissolved microcystin-LR did not affect the fitness of one *Daphnia* clone, but did negatively affect the fitness of another *Daphnia* clone when the effects of this compound are assayed in the absence of other covarying factors (e.g., food size, shape, or nutritional differences). The *Daphnia* clone from Lake Sixteen performed well on the diet containing microcystin-LR (population growth = 0.15) while the clone from Baker Lake showed negative population growth (population growth = -0.09) on the same diet (Fig. 4.1c). These results are intriguing given that the *Daphnia* strain most harmed by microcystin-LR (Baker Lake clone) was the strain that performed better on a mixed diet of 20% toxic *Microcystis* and 80% *Chlorella*. The mixed diet contained microcystins at concentrations (535 μg (g dry weight)$^{-1}$) that were 13x higher then the microcystin-addition diet (42 μg (g dry weight)$^{-1}$). These findings suggest that zooplankton toxicity by microcystins may be influenced by food quality or how microcystins are packaged in cyanobacteria.

The Baker Lake clone performed poorly on freeze-dried *Chlorella*, in general (Fig. 4.1c), and exhibited significant positive population growth for only one diet in either experiment (lipophilic extract addition, *t*-test against a population growth = 0, p = 0.044, Fig. 4.1c). Synergistic effects of the poor food quality of the freeze-dried algal diet and the addition of microcystin-LR could have negatively compromised the health of the Baker Lake clone. The Lake Sixteen *Daphnia* grew well on diets containing freeze-dried *Chlorella* (Fig. 4.1a,c, Tables 4.1 and 4.3), and subsequently, did not show any negative effects of microcystin-LR on growth, maturation rates, and fitness (Fig. 4.1a,c, Fig. 4.2d, Tables 4.1, 4.2, and 4.3). Alternatively, the Baker Lake clone could have been

more sensitive to dissolved microcystin than the Lake Sixteen clone. Particle-bound microcystin concentrations vary widely in nature depending on sample collection time, location, and type (range = undetectable – 7300 μg microcystin (g (dry weight)$^{-1}$ for many lakes varying in trophic status, Chorus and Bartam 1999). Microcystin-LR, a water soluble compound, was not well retained (1% retention; 42 μg microcystin g (dry weight^{-1})) on the freeze-dried algae after rehydration, however the compound was present in ecologically relevant concentrations (median = 106 μg microcystin (g dry weight)$^{-1}$, range = undetectable - 1552 μg microcystin (g dry weight)$^{-1}$, Kotak et al. 1995). The concentration of dissolved microcystin in the microcystin-addition treatment (11.9 μg L^{-1}) was several orders of magnitude lower than concentrations previously shown to harm zooplankton (21400 μg L^{-1}; DeMott et al. 1991; 5000 μg L^{-1}; Ghadouani et al. 2004) and near or below concentrations shown (1) not to reduce the fitness of *Daphnia magna* (3.5 μg L^{-1} , Lürling and van der Grinten 2003) and (2) not to affect the feeding behavior of *Daphnia pulicaria* (50-500 μg L^{-1}, Ghadouani et al. 2004) when fed saturating levels of *Scenedesmus* in the presence of dissolved microcystin-LR. Thus, our findings suggest that any effect of microcystin was through ingestion and not immersion, and that our observation that microcystin-LR can be toxic to some *Daphnia* clones when consumed is conservative. Another explanation could be that the Baker Lake clone sequesters other properties found in live *Microcystis* cells to suppress or counteract the negative effects of microcystin-LR when ingested. Cyanobacteria are composed of thousands of chemical constituents (Stanier et al. 1971), and grazers could sequester compounds that cyanobacteria use to prevent autotoxicity from toxic secondary metabolites. No studies, to date, have addressed this possibility for cyanobacteria-zooplankton interactions (but

see Kainz and Strack 1980; Inderjit and Dakshini 1994; Mai-Prochnow et al. 2004 for autotoxicity studies on other microbes). Additionally, chemical signals produced and received by zooplankton and phytoplankton during grazing have been shown to affect trophic dynamics (Larsson and Dodson 1993; Nevitt et al. 1995; Wolfe 2000). These signals may induce zooplankton to upregulate physiological or behavior responses to lessen the effects of toxic cyanobacteria (Ghadouani et al. 2004). However, such chemical signals, if they exist, might not be present when zooplankton feed on non-cyanobacterial diets containing cyanotoxins. Future experiments using freeze-dried, non-toxic cyanobacteria coated with added cyanobacterial metabolites could address this question. Furthermore, recent studies show that zooplankton can adapt to grazing on microcystin-containing cyanobacteria (Hairston et al. 2001; Sarnelle and Wilson 2005), however our results suggest that these adaptations may be to live cyanobacteria, in general, and not simply to microcystins (but see Gustafsson and Hansson 2004; Gustafsson et al. 2005).

Our findings that microcystin-LR both is and is not toxic to conspecific zooplankton clones is not surprising given that past qualitative (Burns 1987; Lampert 1987; Haney 1987) and quantitative (Wilson et al. in press) reviews of freshwater zooplankton-cyanobacteria interactions provide little consensus on the role of cyanobacterial toxins in affecting zooplankton fitness. In addition, significant interspecific and intraspecific variation exists in the ability of zooplankton to feed and reproduce on different cyanobacterial species and genotypes (Arnold 1971; Hietala et al. 1995; Nandini and Rao 1998). Furthermore, the inherent patchy nature of freshwater habitats and the clonal nature of many zooplankton and all cyanobacteria provide ample

opportunity for simultaneous, ongoing natural selection on adaptive traits of zooplankton and phytoplankton; thus potentially selecting for much variance within and between sites (Hairston et al. 2001; Sarnelle and Wilson 2005; Wilson et al. 2005). Together, these findings suggest that ecological, physiological, chemical, and genetic variation among different cyanobacterial and herbivore genera, species, and genotypes should not be overlooked when making general claims about the toxicity of specific compounds from cyanobacteria to ecologically relevant grazers.

The toxic *Microcystis aeruginosa* strain (UTEX 2667) that we investigated was not chemically defended against *Daphnia* by lipophilic secondary metabolites. In fact, these lipid soluble constituents of *Microcystis* enhanced output of neonates per female for both clones of *Daphnia* (Fig. 4.1b, Table 4.2), despite these *Daphnia* clones having contrasting population growth responses to a diet containing the same strain of *Microcystis*. Moreover, one *Daphnia* strain (Lake Sixteen clone) showed significantly greater population growth on a diet supplemented with the lipophilic extract of a toxic strain of *Microcystis* versus when fed a control diet lacking the extract (Fig. 4.1c, Table 4.3). Cyanobacteria may be deficient in lipophilic constituents, such as highly unsaturated fatty acids, polyunsaturated fatty acids, sterols (Stanier 1971; Ahlgren 1990; von Elert and Wolffrom 2001). When supplemented in the diet, these compounds have been shown to promote increased somatic (DeMott and Müller-Navarra 1997; Weers and Gulati 1997; von Elert and Wolffrom 2001) and population (Martin-Creuzburg et al. 2005) growth of zooplankton. Although hundreds of studies have examined the effects of water soluble secondary metabolites on diverse plants and animals (Zurawell et al. 2005), few studies report the effects of lipid soluble secondary metabolites from freshwater

cyanobacteria (Wheeler et al. 1942; Kurmayer and Jüttner 1999; Keil et al. 2002). Thus, our finding that the lipophilic fraction of *Microcystis* is not detrimental but is instead beneficial for *Daphnia* should not be viewed as surprising. However, we only used one strain of *Microcystis* and two clones of *Daphnia pulicaria* in our study, thus our results are limited. We encourage future experiments designed to evaluate whether phytoplankton commonly, or rarely, produce lipid soluble metabolites that affect herbivorous zooplankton using techniques that test the effects of these compounds through consumption (this study) and not simply immersion (DeMott and Dhwale 1995).

Survivorship and growth rates for one *Daphnia* clone (Lake Sixteen) fed solvent treated freeze-dried phytoplankton were surprisingly high relative to rates on live *Chlorella*, showing that this technique holds promise for identifying chemical components, especially lipid soluble constituents, of cyanobacteria, diatoms, and other phytoplankters, that benefit or harm nonselective filter-feeding herbivores (see Caldwell et al. 2004 for review of alternative techniques). Earlier studies have suggested that *Microcystis* is chemically defended by other water soluble non-microcystin components (Jungmann 1992; Lürling 2003a; Rohrlack et al. 2003); however none of these studies *directly* tested the effects of these specific compounds on zooplankton fitness when consumed. Instead, in these studies, grazers were immersed in media with cyanobacterial filtrates or extracts (Jungmann 1992; Rohrlack et al. 2003) or fed diets containing or lacking microcystin-containing *Microcystis* strains (Lürling 2003a) that may also differ in other co-varying factors. Conclusions from these (Jungmann 1992; Lürling 2003a; Rohrlack et al. 2003) and other studies (Wheeler 1942; Shelubsky 1950; Kurmayer and Jüttner 1999) support the notion that other non-microcystin compounds may be harmful

to zooplankton; however more conclusive tests need to be conducted using alternative techniques provided in this study (especially for lipophilic compounds) or elsewhere (Caldwell et al. 2004) before definitive conclusions can be drawn about the roles of cyanobacterial secondary metabolites on zooplankton fitness.

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